Expression of the Protective Antigen of *Bacillus anthracis* in attenuated *Salmonella*: a potential oral anthrax vaccine.

by

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Publications

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Declaration

I hereby declare that this thesis was composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record was performed by myself unless otherwise stated, and that all sources of information have been specifically acknowledged.

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Summary

Anthrax is a disease of animals and man caused by *Bacillus anthracis*. The Protective Antigen (PA) of *B. anthracis* can induce protective immunity and is a candidate vaccine antigen, but current vaccines are not ideal. Live vaccine vectors, such as the aromatic amino acid *Salmonella* mutants, are capable of delivering antigens to the immune system and stimulating immune responses. The aim of this project was to develop a recombinant *S. typhimurium* expressing PA and to evaluate the protective immune responses generated in mice.

Native PA was expressed at low levels in *S. typhimurium*. Various approaches to increasing expression were tried including changing the promoter, expressing the 63 kDa C-terminal fragment of PA as a fusion protein and expressing this fragment after a signal sequence. Altering the *B. anthracis* PA promoter to the *E. coli lac* promoter increased expression of PA which was exported with the *B. anthracis* signal sequence. The cytoplasmically located PA fragment was unstable and was not successfully exported by the signal sequence.

The recombinant organisms were evaluated to select one for *in vivo* study. Many phagemids were unstable without ampicillin selection and an attempt to stabilise one with the *cer* region was not successful. Two constructs were chosen for animal work.

Recombinant PA was shown to induce partial protection, comparable to native PA, when both were purified and administered with adjuvant. Mice were vaccinated intravenously with the live *S. typhimurium* constructs and subsequent challenged with virulent *B. anthracis* spores. The PA-expressing-*S. typhimurium* only colonised at low levels but induced partial protective responses. These protective responses occurred without detectable anti-PA antibody.

This work showed that PA expressed by *S. typhimurium* can induce protective responses even when only low colonisation occurs. It shows the potential for this approach and suggests that further work to stabilise and increase the expression of PA would be worthwhile.

Abbreviations

aa	amino acid
Abs	Absorbance
ABTS	2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid)
Amp^r	Ampicillin resistant
Amp^s	Ampicillin sensitive
APC	Antigen presenting cell
ATP	Adenosine triphospahte
bp	base pairs
CAMR	PHLS Centre for Applied Microbiology and Research, Porton Down. Formerly MRE
CBDE	Chemical and Biological Defence Establishment, Porton Down
DAB	3,3'-diaminobenzidine
DHA	2,3 dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminotetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
FIA	Freunds incomplete adjuvant
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscularly
IP	Intraperitoneally
IPTG	Isopropylthiogalactoside
IV	Intravenously
kbp	kilo base-pairs
kDa	kiloDaltons
LB	Loading Buffer
LT-B	*E. coli* Heat-Labile Toxin, B subunit
MAb	Monoclonal antibody
MHC	Major histocompatibility complex

MOMP	Major outer membrane protein
MRE	Microbiological Research Establishment, Porton Down. Now CAMR
mRNA	messenger RNA
MW	Molecular weight
nt	nucleotide
OMP	Outer membrane protein
ORF	Open Reading Frame
ori	origin of replication
PA	Protective Antigen
PABA	p-aminobenzoic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PVDF	Polyvinyldifluoride membrane
RBS	Ribosome Binding Site
RES	Reticuloendothelial system
RNA	Ribonucleic Acid
rPA	recombinant Protective Antigen
SC	Subcutaneously
S/D	Shine Dalgarno region
SDS-PAGE	Sodium dodecyl sulphate - PAGE
ssDNA	single-stranded DNA
TIR	Translation Initiation Region
TPCK	N-Tosyl-L-phenylalanine chloromethyl ketone
tRNA	transfer RNA
ttd	time to death
USAMRIID	US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA.
UV	Ultraviolet
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

Introduction

1.1 Anthrax

Anthrax is an infectious disease known since antiquity. It is thought to be responsible for the fifth plague of Egypt and more recently in 1613 the 'Black bain' plague in Europe in which 60,000 people are believed to have died (Whitford, 1979). It is nearly universal in its geographic distribution and, although primarily a disease of herbivores, it can affect many species including man (Christie, 1987). Accurate figures on the incidence are hard to obtain as most cases occur in under-developed regions (Fujikura, 1990). In 1985, of 168 countries supplying data to the Food and Agriculture Organization, 83 reported bovine anthrax, 56 ovine, 25 equine and 21 porcine (Turnbull, 1990). Comprehensive recent global figures on human cases are not available (Fujikura, 1990). World Health Organisation (WHO) reports use the estimates of Glassman (cited in Whitford, 1979) of 20,000 to 100,000 human cases per year. In Zimbabwe a massive outbreak occurred during 1978-80 with 9711 human cases and 151 deaths. Even in Europe there was an average of 429 human cases per annum during the period 1979-84 (Turnbull, 1990). Anthrax can now be controlled in man and domesticated animals by the use of vaccines, but in wild herbivores it still causes major epidemics. In endangered species anthrax can have a significant effect. In the Etosha National Park, Namibia, anthrax was thought to be responsible for the decline in numbers of the blue wildebeest from 30,000 in 1965 to 2,500 in 1978. Between 1959 and 1962 approximately 25% of the South African population of roan antelopes (estimated at 320) were killed by anthrax (Turnbull, 1990). The WHO have a Working Group monitoring anthrax disease control worldwide.

1.1.1 *Bacillus anthracis*

Anthrax is caused by ***Bacillus anthracis***, a Gram positive, non-motile, facultatively anaerobic spore-forming organism. The vegetative bacteria, when recovered from infected animals, are rod-shaped 3-8 μm long and enclosed by a thick polypeptide capsule visible under the microscope with certain stains (Christie, 1987). In the presence of oxygen sporulation occurs. The spores are ellipsoid, non-bulging and central. There is a considerable difference between the resistance of the vegetative and spore forms. The vegetative cells are easily killed by heating to 55°C for 1 hour, and by most disinfectants and antibiotics. In an unopened carcase the vegetative cells are destroyed during putrefaction within 80 hours at summer temperatures (Christie, 1987). On the other hand the spores of *B. anthracis* are

extremely resistant to heat, cold, ultraviolet light, desiccation, high and low pH, chemical disinfectants and the products of other bacteria (Turnbull, 1990). For instance, they can survive 160°C dry heat for one hour, 68 years in the dry state and up to 60 years in soil. Moist heat is more effective and autoclaving at 121°C for 15 mins will kill all spores (Christie, 1987).

1.1.2 Anthrax in Animals

Herbivores usually acquire the disease by ingesting spores from contaminated soil. This leads to the gastro-intestinal form of the disease where the spores germinate within a few hours and the bacteria rapidly develop a capsule (Turnbull, 1990). A local lesion forms with considerable oedema. In susceptible animals the bacteria multiply and invade the blood leading to bacteraemia, toxaemia and death. The bacilli are found in large numbers in the blood and tissues of the animals and, if the bacteria become exposed to the air (in discharges from the animal or due to scavenger attack), they sporulate and contaminate the local area (Turnbull, 1990). In herbivores the disease is often hyperacute and the animals may be found dead. Animals may also be infected through the skin via wounds or by biting insects.

Other species are more resistant to anthrax, the course of infection may be longer and oedema may be more pronounced. Carnivores are particularly resistant and may often have antibody to anthrax toxin suggesting recovery following infection (Turnbull, 1990).

Contaminated feedstuffs or fertilizers and industrial waste from the wool and leather industries are also important in the epizootiology of the disease (Christie, 1987).

1.1.3 Anthrax in Man

Anthrax in man is often classified as either industrial or non-industrial. Industrial anthrax arises from contact with contaminated animal products. Hides, skins, wool, hair, bones, hooves and horn are all implicated. Non-industrial anthrax occurs in farmers, pathologists and veterinarians as a result of close contact with infected animals (Christie, 1987).

The disease takes one of three forms (Knudson, 1986). Most human cases are of cutaneous anthrax contracted from direct contact with infected material and show the development of the characteristic black eschar or carbuncle. The word anthrax is derived from a Greek word meaning charcoal (Whitford, 1979). With treatment most cutaneous

cases survive. Pulmonary anthrax is usually caused by industrial exposure to aerosolized spores. The spores are aerosolized during handling of contaminated material such as hides and hair. Illness begins 2-5 days after exposure and within hours the patient is acutely ill. Death occurs in 2-3 days and treatment is usually unrewarding once clinical signs develop (Hambleton *et al.*, 1984). The third form is intestinal anthrax and this is very rare. It usually results from eating meat from anthrax carcases (Lakshmi & Kumar, 1992). The eschar develops within the gastrointestinal tract and, if diagnosed early enough, treatment can be successful (Knudson, 1986).

1.1.4 Anthrax Virulence

The virulence of *B. anthracis* is attributed to two factors, the polypeptide capsule and a tripartite exotoxin. Virulent strains contain two large plasmids pXO1 (170-185 kbp) and pXO2 (90-95 kbp) (Leppla, 1991). The genes for the toxin components (see Section 1.1.5) are found on pXO1 and the capsular genes (*capA,B* and *C*) are on pXO2. *B. anthracis* strains vary in their virulence not only based on the presence of these plasmids but also between strains containing both plasmids. It is not clear if the difference is due to genetic variation between the plasmids, or if the strains have chromosomal differences. Chromosomal elements can affect virulence, possibly by regulating toxin or capsule expression from the plasmids (Welkos, 1991).

The gamma-linked poly-D-glutamic acid capsule is thought to play a role in the establishment of the infection. Capsulated strains resist phagocytosis *in vitro* (Whitford, 1979) and insertional inactivation of the *cap* genes increases the ability of neutrophils to phagocytose the bacilli (Welkos, 1991). Regulation of the expression of the capsule is complex and affected by regions both on the plasmid containing the *cap* genes and the chromosome (Welkos, 1991). Capsule production is influenced by the medium composition and CO_2 level that the culture is grown in (Bartkus & Leppla, 1989). Encapsulated, non-toxigenic strains are attenuated but do not induce protective immune responses, whereas non-encapsulated, toxigenic strains are also attenuated but do induce protective responses (Ivins *et al.*, 1986). Therefore the capsule is not considered an important protective antigen.

The role of anthrax toxin is less clear but is generally accepted to be the cause of death (Leppla, 1991). The pioneering work in this field was carried out during the 1950s and 60s. It was shown that if one administered streptomycin to guinea pigs infected with

B. anthracis, the animals could be saved if the antibiotic was given early enough. If the antibiotic was given later in infection, even though the blood could be sterilized , the animals would still die (Smith & Keppie, 1954). Smith also showed that sterile plasma obtained from animals dying of anthrax was lethal when injected into mice and guinea pigs. It was then shown that the bacteria-free extracellular products of *B. anthracis* were immunogenic (Gladstone, 1946). Over the next 10-15 years the complex nature of the toxin was identified, although many questions were raised as to the mechanism of action (Smith & Stoner, 1967). Even after 30 years, the answers to some of these questions is still awaited (Leppla, 1991).

As the toxin plays a central role in both virulence and protection it is discussed in more detail.

1.1.5 Anthrax Toxin

The anthrax toxin is composed of three proteins, Protective Antigen (PA), Lethal Factor (LF) and Oedema Factor (also spelt Edema Factor, EF). The genes for all three have been cloned and sequenced (Welkos *et al.*, 1988; Bragg & Robertson, 1989; Escuyer *et al.*, 1988). PA is the cell binding component which promotes the internalization of either LF or EF. The combination of PA and LF is referred to as Lethal Toxin, and PA and EF is called Edema Toxin. Edema Toxin causes oedema when injected intradermally in animals and EF is a calmodulin dependent adenylate cyclase (Leppla, 1982). Edema toxin has an obvious role in creating the extensive oedema seen in some manifestations of the disease and also has a direct effect on neutrophils and this may inhibit the mobilization and activation of phagocytes critical for the clearance of the bacteria (Leppla, 1991). When injected intravenously in susceptible animals Lethal Toxin causes rapid death but its mechanism of action is unknown although it is assumed to have an enzymatic action within the cytosol (Leppla, 1991; Friedlander *et al.*, 1993). LF has been reported to have an homologous region to the zinc-binding site of metalloproteases (Klimpel *et al.*, 1993). Lethal toxin may help to establish the infection by incapacitating phagocytes and causing cell death and is considered responsible for the lethal action of anthrax toxin (Leppla, 1991).

B. anthracis produces low yields of toxin in most rich media, whereas bacteria grown in certain minimal media produce increased levels of toxin if bicarbonate is added and the culture is grown under CO_2 (Bartkus & Leppla, 1989). Trans-acting factors on plasmid pXO1 are required for regulation of the transcription of the PA gene (Bartkus & Leppla,

1989) and maximal production occurs in early stationary phase (Cataldi *et al.*, 1992). Lethal toxin is produced from early logarithmic through stationary phases when cultured *in vitro* (Ezzell *et al.*, 1984).

A model for the action of anthrax toxin has been proposed (Leppla, 1991) and is illustrated in Figure 1.1. PA is secreted from *B. anthracis* as an 83 kDa protein (PA-83). PA-83 binds to a specific cell surface receptor where it is cleaved by a trypsin-like protease into 63 and 20 kDa fragments (PA-63 and PA-20). PA-63 remains bound to the receptor and loss of PA-20 reveals a specific binding site for which the secreted EF and LF compete. The PA-63 in a complex with either EF or LF is internalized by endocytosis and LF or EF is transferred into the cytosol where they have their toxic action. Although this model may be applicable to some tissue areas, evidence exists that the PA-83 becomes cleaved by a serum protease and can form complexes of PA-63 and LF prior to cell binding. It is suggested that a pentamer of PA-63 may complex with each LF molecule (Ezzell & Abshire, 1992).

Owing to the central role of PA in the toxic action, and the fact that it is non-toxic itself, PA is recognised as an important antigen in vaccine production.

1.1.6 Physical Control Methods

Apart from vaccination, anthrax cases in both animals and man can be reduced by minimising exposure to *B. anthracis* spores (Christie, 1987). The carcases of animals which have died of anthrax are the main sources of spores and disposal of carcases is defined by law in many countries. As long as the carcase is unopened the *B. anthracis* will not sporulate thus post-mortems should not be carried out. The carcase should be buried with quicklime or incinerated. Proper carcase disposal is expensive and may not be practical in developing countries. It is also difficult to arrange for wild animal populations. Once a pasture is contaminated, it is time-consuming to decontaminate it. Chemicals such as formaldehyde have been used for this purpose (Manchee *et al.*, 1983), but in general susceptible animals should be kept from grazing on contaminated pasture.

For man, infection is usually acquired from contaminated animal by-products. Reducing exposure to spores has led to a decrease in human cases. This is usually accomplished by sterilising contaminated products such as raw bones, hair and wool. Disinfection of imported wool was carried out at a Government Wool Disinfection Station

Figure 1.1 Model for the action of anthrax toxin on cells

The diagrams show one model for the binding and internalization of anthrax toxin into a cell. From Leppla (1991).

A. PA is secreted from *B. anthracis* as an 83 kDa protein. PA binds to a specific cell-surface receptor.

B. One model has 83 kDa PA cleaved on the cell surface by a trypsin-like protease into 63 and 20 kDa fragments. Cleavage of the 20 kDa fragment reveals a specific binding site for which the secreted EF and LF compete.

C. LF or EF binds to 63 kDa PA and then it is internalized by endocytosis and LF or EF is transferred into the cytosol.

Facing page 7

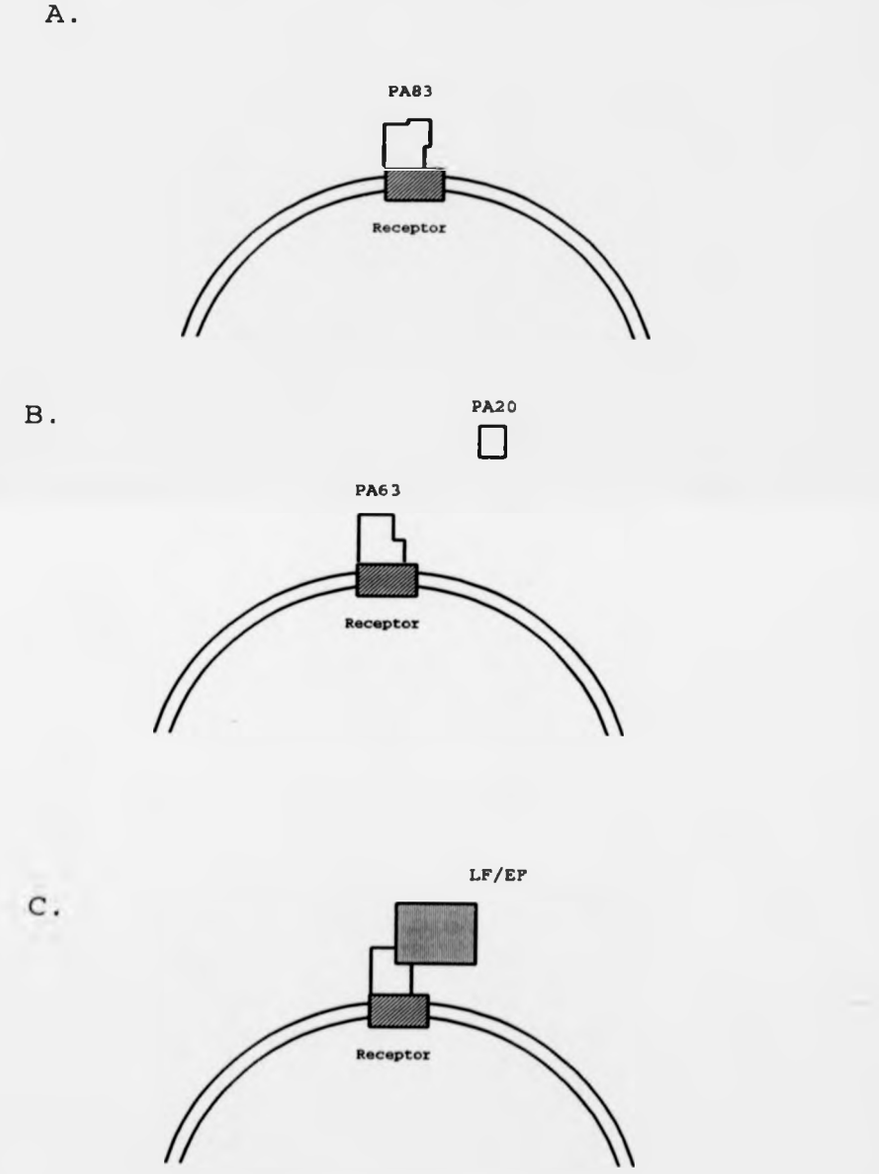

Figure 1.1 Model for the action of anthrax toxin on cells

in Liverpool from 1921 and credited with a large reduction in human cases (Whitford, 1979). Meat from infected animals should not be consumed although this often occurs in third world countries (Turnbull, 1990; Lakshmi & Kumar, 1992).

Physical control methods are expensive to implement and so may not be practical in developing countries. The cost-benefit of large scale disinfection of bulk raw materials must be carefully assessed (Christie, 1987). Wild animal populations cannot be protected by proper carcase disposal and much of the pasture used by animals in Africa is contaminated (Turnbull, 1990). In these circumstances other methods of control, such as vaccination, may provide a better solution.

1.1.7 Anthrax Treatment

Penicillin is the drug of choice and most isolates of *B. anthracis* are susceptible to this antibiotic (Turnbull, 1990). As cutaneous anthrax has a chronic course, oral treatment is usually successful. In pulmonary anthrax more aggressive therapy must be given as, once symptoms develop, treatment is often unsuccessful (Knudson, 1986). Successful management of intestinal anthrax depends on early diagnosis. If for any reason penicillin is not indicated, then tetracyclines, erythromycin or streptomycin can be used. Anti-anthrax serum was advocated before antibiotics became available. The serum was raised in horses, goats, donkeys, cattle and sheep (Knudson, 1986). Up to 1 litre of anti-anthrax serum was given and anaphylactic reactions were common.

1.1.8 Anthrax Vaccines

Animal anthrax vaccines were first introduced on a wide scale by Pasteur in 1881 (Turnbull, 1991). His vaccine consisted of live spores, partially attenuated by culture at elevated temperatures. This cures a proportion of the bacteria of plasmid pXO1, although it is now believed that the immunogenicity of this vaccine is attributable to the small number of toxigenic bacteria which were not cured of plasmid pXO1 (Whitford, 1979). Saponin was added to provoke a violent immune response. These early vaccines still retained some virulence and varied from batch to batch. In 1937 Sterne developed an attenuated live spore vaccine which still remains in use today (Hambleton *et al.*, 1984). His strain ($34F_2$) had been cured of the pXO2 plasmid and so did not produce a capsule but was still toxigenic. Its wide veterinary use throughout the world has dramatically reduced outbreaks of anthrax

in domesticated animals and contributed to a reduction in human cases by reducing human contact with contaminated animal products (Turnbull, 1991).

In Russia a live spore vaccine derived from Sterne's strain has been used in man, but live spore vaccines are not considered safe enough for human use in the West. Live spore vaccines can vary from batch to batch, are contraindicated in many medical conditions and require careful use (Turnbull, 1991). In the 1950s the UK developed an alum-precipitated cell-free filtrate of Sterne strain cultures grown so as to maximise the PA content. In the 1960s the US developed a similar aluminium-hydroxide adsorbed vaccine (Hambleton *et al.*, 1984). The PA is considered to be the major antigen in these vaccines. These vaccines are administered by multiple parenteral vaccinations and cause side-effects ranging from local to systemic reactions. It is difficult to assess the impact of these human vaccines on the reduction of anthrax cases as they were introduced at the same time as improved industrial practices were reducing the level of anthrax cases. One study did find the alum precipitated vaccine to be 92.5% effective in an industrial setting (Brachman *et al.*, 1962). Animal studies have however suggested that these vaccines are not fully effective against challenge with large doses of virulent organisms, particularly by the aerosol route (Turnbull, 1991).

Satisfaction with the current veterinary vaccine used in domesticated animals is not matched with regard to the human vaccine. The long course of primary injections, need for annual boosters, side-effects and concerns about the protective efficacy of the vaccine have all encouraged research into new vaccines (Hambleton *et al.*, 1984; Turnbull, 1991). There is also a need for an alternative vaccine for use in wild animals, particularly in Africa, where anthrax is a major problem in some areas and a significant killer of endangered species. Although it is possible to administer the current veterinary vaccine by darting individual animals with a syringe projectile containing vaccine, this is costly and can only be used in certain restricted areas. Ideally, a cheap oral vaccine which can be administered in food bait is required (Turnbull, 1991).

1.2 Vaccine Requirements

The ideal vaccine would be a single dose oral vaccine giving life-long protective immunity without side-effects. It should be cheap to produce and stable in storage. Originally there was little pressure to develop a new human vaccine. The current vaccines are adequate in controlling industrial anthrax when improved industrial safety measures are

also in effect. The level of anthrax in domesticated animals is controlled by the live spore vaccine and so reduces the infectious burden in animal products and thus the exposure risk to man. Although it is considered unsatisfactory to administer crude and undefined preparations such as the culture supernatant used in the human vaccines, there was little pressure to develop a new vaccine until, in the 1980s, Western countries became concerned about the potential use of an aerosol of virulent anthrax spores as a Biological Warfare weapon. Concerns about the efficacy of the current vaccines against such challenges and the other problems associated with the current vaccines led to much research on anthrax and potential replacement vaccines. The increasing interest in wild life conservation also directed funding towards a vaccine solution for African wildlife.

1.2.1 Experimental Work

During the 1980s the toxin components were cloned and sequenced (Welkos *et al.*, 1988; Bragg & Robertson, 1989; Escuyer *et al.*, 1988). The genetics of *B. anthracis* were clarified and a number of conclusions reached. It was shown that the toxin, or part of the toxin, was required for protective immunity. The Sterne strain does not possess the capsule but can induce high levels of protective immunity (Turnbull, 1991). If strains of non-toxigenic *B. anthracis* were used as live vaccines they conferred no protection (Ivins & Welkos, 1988). PA was found to be the main immunogen and animal data has shown that a strong immune response to PA alone can protect against challenge with even the most virulent strains (Ivins & Welkos, 1988). Finally, it has been shown that protective immunity is not solely a function of anti-PA antibody levels as immunity to anthrax, following Sterne spore vaccination, can occur in the presence of low anti-PA titres (Ivins *et al.*, 1990; Turnbull, 1991). Two possibilities for the protective mechanism have been suggested. Firstly cell-mediated immunity (CMI) may be important as adjuvants that are known to stimulate CMI enhance the protective effect of PA vaccines (Ivins *et al.*, 1992). The aluminum compound adjuvants used in the human PA vaccines are known to augment the humoral response and not the CMI (Gupta *et al.*, 1993) and this failure to stimulate CMI may be responsible for their lack of effectiveness. Secondly, current PA vaccines may not stimulate the protective class of antibodies produced by the Sterne strain. The UK and US vaccines may not present PA to the immune system in the native conformation. The work of Ezzell & Abshire (1992) has suggested that PA may be complexed to LF in the serum.

The epitopes expressed by this complex may be quite different to that of adsorbed or precipitated PA. Thus, although the anti-PA antibody titre may be lower following live strain vaccination, the antibodies present may have a higher affinity for the natural toxin complex. This possibility is considered less likely as strongly adjuvanted PA alone can be protective.

The work described above suggests that a suitable vaccine could be made using PA alone, but it must be presented to the immune system such that protective responses are stimulated.

1.2.2 Approaches to new vaccines

There are three main approaches to developing a new vaccine.

Firstly there is the possibility of a subunit vaccine based around PA. Some of the unpleasant side-effects of the current vaccine may be attributable to components other than PA in the vaccine and so a purified PA vaccine may be less reactogenic. Strongly adjuvanted PA has been shown to confer protective immunity in animals but the adjuvants used are not suitable for humans and produce severe side-effects. Various novel adjuvants have been tried such as TriMix or Detox (Ribi Immunochem Research Inc., Hamilton, Montana, USA) and these have produced good immunity but there are still concerns about their toxicity (Turnbull, 1991). It may prove difficult to separate the strong adjuvanting effects of these compounds from their toxic effects. Much research is taking place in this area and a suitable novel adjuvant may become available. PA subunit vaccines may give better protection if the PA is configured in the native form. If, as suggested by Ezzell & Abshire (1992), this is as pentamers with a single LF molecule, it may be possible to genetically engineer LF so that it is non-toxic but can still bind to PA stably and form the naturally configured complex. This complex may stimulate a more protective class of anti-PA antibody than PA alone. A subunit vaccine of this type might also have the advantage that it would be simpler to licence than a live vaccine.

The second approach is to rationally attenuate *B. anthracis* so as to benefit from the success of the Sterne animal vaccine, but with a higher degree of safety that is required for a human vaccine. An auxotrophic mutant of the Sterne strain has been constructed and tested in animals and found to confer strong protective immunity (Ivins *et al.*, 1990). Such an approach has the advantage of presenting the full range of the anthrax antigens (less the

capsule and pXO2 associated proteins) to the immune system. Although PA has been shown to induce protective immunity under the right circumstances, other antigens may contribute to immunity although efforts to identify such antigens have failed (Ezzell & Abshire, 1988). It would of course be necessary to ensure that the mutant selected could not revert to virulence by using several deletions that attenuate independently. Such a mutant might not be orally active as the rarity of the intestinal form of anthrax is probably related to its low infectivity by this route. Therefore a rationally attenuated *B. anthracis* vaccine might have to be administered by injection or scarification.

A third approach, suggested by Winther & Dougan (1984), is to use a live carrier vector to target PA to the immune system. Vaccine vectors are used to express heterologous gene products. When the vector is administered, not only is there the possibility of an immune response to the vector, but also to the heterologous protein. As it is known that strongly adjuvanted PA alone can protect against anthrax, and the gene for PA is available, this approach is practical. PA has been expressed in the related bacterium *B. subtilis* and 10^{10} live spores were able to protect guinea pigs against IM challenge with Ames strain *B. anthracis* (Ivins *et al.*, 1990). This result illustrates the potential for a vector approach. If the vector was active orally then this means of administration becomes practical. The vector used could have important consequences for the nature of the immune response generated. The vector should be chosen to stimulate those arms of the immune system that are required for protective immunity.

Although subunit vaccines have many advantages over live vaccines, a suitable adjuvant has not been identified that can stimulate a strong enough response to PA. An attenuated *B. anthracis* might induce protective immunity but is unlikely to be orally active. As PA has been identified as the only antigen needed for protection, it is a candidate antigen for expression from a vaccine vector. Living vectors can stimulate strong immune responses and some vectors are orally active and so might ease vaccine administration. Therefore the last approach offers the best potential of achieving the ideal vaccine requirements.

1.3 Vaccine Vectors

The suitability of many bacteria and viruses as vaccine vectors has been investigated over the past decade. In many cases organisms have been considered as vaccine vectors on the basis of the ease with which genetic manipulations could be carried out. Other factors

are important and include: a full understanding of the organism so that rational decisions can be taken over its attenuation and use. its ability to carry and express foreign genes and configure and present the protein antigens appropriately. the safety of the constructs in relation to side-effects and reversion to virulence. and the ability of the organism to induce appropriate protective immune responses.

Some of the organisms that have been considered as vaccine vectors are shown in Table 1.1. One of the desirable features of a new vaccine is oral administration. As PA is a bacterial antigen it may be more logical to select a bacterial vector. The most promising orally active bacterial vector is attenuated *Salmonella*.

1.3.1 Attenuated *Salmonella*

When administered orally to mice *S. typhimurium* invade via the M cells of the Peyers patches before moving to the Mediastinal Lymph nodes and then to the liver and spleen where they can establish an infection in the reticuloendothelial system (RES) (Carter & Collins, 1974). If an attenuated strain is used. then this infection is cleared after several weeks, and the animals remain immune to infection with wild type strains (Killar & Eisenstein, 1985; Dougan *et al.*, 1988).

In the search for safe live vaccines against Salmonellosis, attention has focused on mutant strains which have decreased virulence. The decreased virulence can often be associated with the loss of a plasmid or a defective gene. There are two approaches to the development of attenuated organisms with defective genes. Firstly it is possible to generate random mutations, select for reduced virulence and then investigate the nature of the mutation (Edwards & Stocker, 1988; Finlay *et al.*, 1988). A second approach is to construct organisms with specific defects in their genetic material and evaluate the effect. These two approaches have identified potential vaccine candidates and some are listed in Table 1.2.

Plasmidless mutants of some *Salmonella* strains may have reduced virulence, but other strains, such as *S. typhi*, do not have a virulence plasmid. Auxotrophic mutants are of varying virulence. This allows the degree of attenuation to be selected as required. The aromatic amino acid mutants have been extensively studied and are promising candidate strains. This class of mutants is discussed in detail below. Invasion/survival in eukaryotic cell mutants have yet to be fully characterised. but have the potential to allow a precise degree of invasion before the mutation prevents further steps in the normal invasion cycle.

Organism	Comments	Reference
Vaccina virus	Extensively studied, may lead to generalised infection in immunocompromised individuals.	Moss. (1991)
Avipox viruses	Cannot replicate in mammalian cells. Potentially safer than Vaccinia.	Anon. (1992)
Adenovirus	Administered orally to US Army recruits.	WHO. (1990)
Poliovirus	Only short epitopes expressed.	WHO. (1990)
E. coli	Stimulate surface immunity unless invasion competent.	WHO. (1990); Guzman, (1991)
S. typhimurium	Animal model for *S. typhi* in humans, orally active, extensively studied.	Formal *et al.*, (1981); Curtiss. (1990); Hormaeche (1991)
S. typhi	Attenuated strains as vectors for humans.	Cryz. (1992)
Listeria monocytogenes	Intracellular pathogen, attenuated strains being developed, β-galactosidase expressed and induces weak responses.	Schafer *et al.*, (1992)
Yersinia enterocolitica	Orally active, poor protection against *Yersinia* challenge but induce antibody to expressed I.T-B.	O'Gaora, (1990)
BCG	Stable, safe, low-cost and good cellular immunity, difficult to manipulate.	Stover *et al.*, (1991)
Streptococcus gordonii	Commensal organism of the oral cavity.	Pozzi *et al.*, (1992)
Corynebacterium pseudotuberculosis	Attenuated strain proposed as live vector.	Hodgson *et al.*, (1992)

Table 1.1 Candidate vaccine vectors

Class	Attenuation	Comments
Plasmidless mutants	Virulence plasmid cured	Have decreased virulence but can still cause disease (Jones *et al.*, 1982).
Auxotrophic mutants	*asd* (aspartate β-semialdehyde dehydrogenase) mutations	Require diaminopimelic acid and are avirulent but only induce a generalized secretory immune response and little or no humoral or cellular immunity (Curtiss *et al.*, 1987).
	Thymine-dependent mutations	Of reduced virulence only (Curtiss, 1990).
	Purine mutants	Level of attenuation depends on where in the pathway the mutation is located. Mutations before the inosine monophosphate branch point are only partially attenuated, and those after may be hyperattenuated. (Sigwart *et al.*, 1989; O'Callaghan *et al.*, 1988).
	Aromatic amino acid mutants	Mutations in the aromatic amino acid biosynthesis pathway. These are discussed below in detail.
	Others including *his*, *cys*, *pncB*	Of limited use (Curtiss, 1990)
Invasion/survival in eukaryotic cells	Mutants defective in transcytosis or cell attachment	Tn*phoA* mutants, several groups, attenuated to varying degrees (Finlay *et al.*, 1988)
	Macrophage cell-line survival	Mutations affecting survival within the macrophage (Fields *et al.*, 1986).
Antibiotic associated mutations	Streptomycin-dependent	High reversion frequency, poorly immunogenic, sensitive to lyophilization (DuPont *et al.*, 1970; Levine *et al.*, 1976).
	Metabolic-drift mutations	Nal/Rif/Hat stable strain used as a Russian sheep vaccine (Linde *et al.*, 1992)
Temperature-sensitive mutants	'Ts' counter mutants	High reversion frequency and not fully characterised (Ohta *et al.*, 1987).
Carbohydrate metabolism mutants	*galE* mutations.	Galactose epimerase-less mutants which form basis of *S. typhi* Ty21a vaccine strain. A complex and undefined mutation whose attenuation may not be due to the *galE* defect (Germanier & Furer, 1975; Hone *et al.*, 1988a).
Global regulator mutants	*cya*, *crp* mutants	Mutations in the cyclic AMP pathway, a key regulatory system necessary for the transcription of many genes. The double mutation provides security against spontaneous reversion. A promising system of attenuation. (Curtiss & Kelly, 1987)
	ompR	*ompR* regulates expression of genes including outer membrane proteins OmpC and OmpF. *ompR* mutants are attenuated (Dorman *et al.*, 1989). Lack of these OMPs is not responsible for the attenuation (Chatfield *et al.*, 1991).
	phoP	Two-component system responsive to phosphate levels, regulates several virulence-associated genes, attenuated in mice (Miller *et al.*, 1989a; Miller & Mekalanos, 1990).
Other virulence associated mutants	*htrA*	Serine-protease homologous to *E. coli* heat shock protein, attenuated in mice (Johnson *et al.*, 1991; Chatfield *et al.*, 1992)
	hemA	Mutants unable to carry out oxidative respiration, attenuated and immunogenic in mice (Benjamin *et al.*, 1991)

Table 1.2 Potential *Salmonella* vaccine candidates.
Based on Curtiss, (1990) and Chatfield *et al.*, (1992)

The antibiotic-associated mutants are often point mutations which can revert at high frequencies. Although they may be suitable as animal vaccines, this method of attenuation is unlikely to be approved for human use. The temperature-sensitive mutants also revert at high frequencies and the mutations are not fully characterised. The carbohydrate metabolism mutants have an extensive history. The *galE* mutation was thought to be responsible for the attenuation of the *S. typhi* vaccine strain Ty21a. Subsequent work has shown that other mutations in this strain are responsible for the attenuation. Global regulator mutants can be highly attenuated as the organism is unable to respond effectively to changes in its environment as it invades the body. There is some concern that, as the actual gene responsible for virulence is not deleted, escape mutants could occur which could revert to virulence. A safer approach is to identify the virulence-associated gene and delete all or part of the gene. Promising candidates include the *htrA* mutants. It may be possible to delete several virulence-associated genes to achieve the desired level of attenuation.

1.3.2 Aromatic Amino Acid Mutants

In 1950 Bacon and co-workers (Bacon *et al.*, 1950a; Bacon *et al.*, 1950b) showed that some mutant strains of *S. typhi*, induced by chemicals and radiation, had reduced virulence for mice and would only grow in the presence of particular substrates. One of these auxotrophs required p-aminobenzoic acid (PABA). Subsequently the aromatic biosynthesis pathway has been elucidated. Mutations in this pathway lead to a requirement for aromatic compounds and reduced virulence. The pathway is shown in Figure 1.2 (from Stocker, 1988). Evaluation of *aro* mutants as possible candidates for attenuated live *Salmonella* vaccine strains has been undertaken.

The first *aro* mutation constructed using modern genetic techniques was in the *aroA* gene in *S. typhimurium* (Hoiseth & Stocker, 1981). 2,3-dihydroxybenzoic acid (DHA), PABA, and the three aromatic amino acids, are required for growth of *aro* mutants. The three aromatic amino acids are available in the host tissues, but PABA and DHA are not available. PABA is required for folate production and DHA for enterochelin. Although an enterochelin mutant alone causes some attenuation (Yancey *et al.*, 1979), the folate deficiency is thought to be more important. The mechanism by which this deficiency causes attenuation is not clear but it has been proposed that the lack of folic acid leads to a depletion of formyl-methionine and thus an inability to initiate new protein chains (Stocker,

Figure 1.2 Aromatic Amino acid pathway

The diagram shows the main components of the aromatic amino acid pathway. The genes which code for enzymes involved in each step are shown. Chorismic acid is required for many synthetic products. For the purposes of *in vivo* attenuation all except PABA and DHB are available from the host and so it is the depletion of these compounds, and their derivatives, that is held responsible for attenuation.

PEP	P-enolpyruvate
EP	Erythrose-4-P
DAHP	3-deoxy-D-arabino-heptulosonic acid 7-P
DHQ	Dehydroquinate
DHS	Dehydroshikimate
SAP	Shikimate-3-P
EPSAP	Shikimate-5 enolpyruvate 3-P
PABA	Para-aminobenzoic acid
DHB	2-3 dihydroxybenzoic acid

Facing page 17

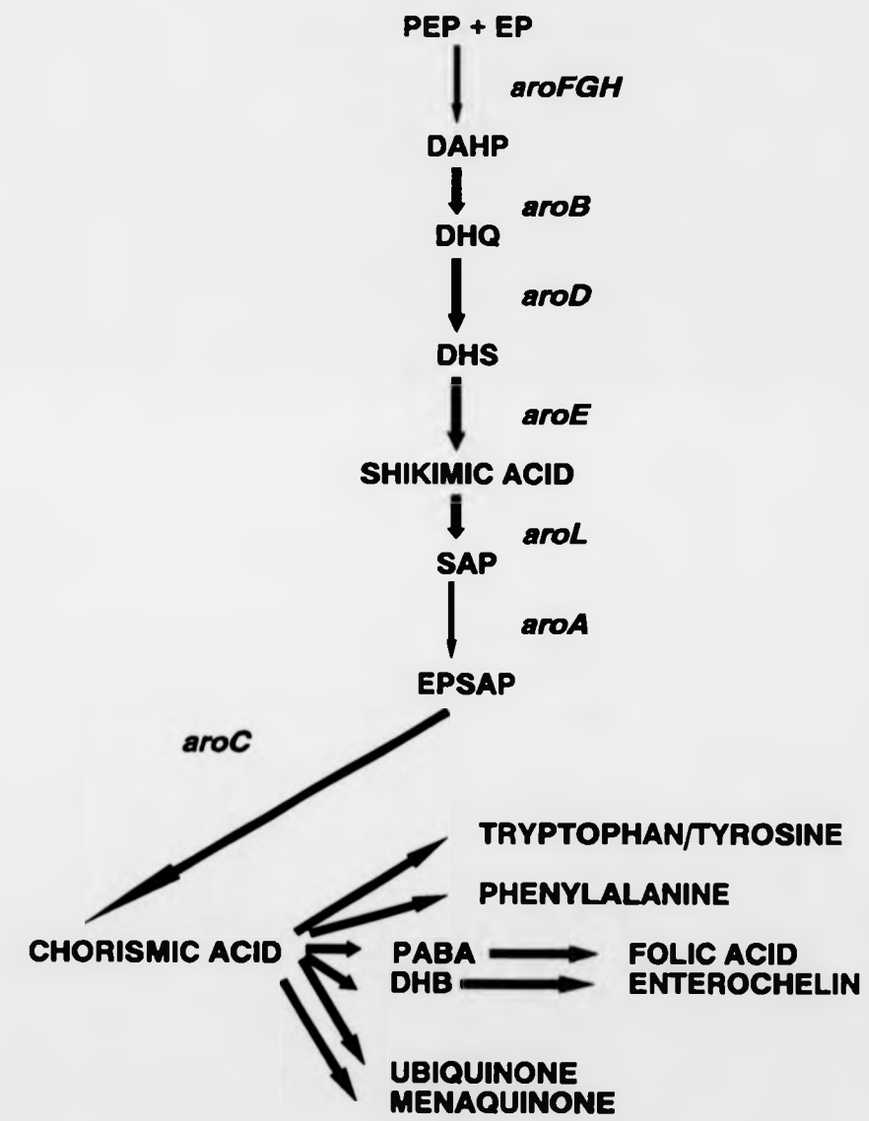

Figure 1.2 Aromatic Amino acid pathway

1990). The doubling time in the mouse spleen for a *S. typhimurium aroA* mutant is 3.4 days as compared to 9.9 hours for a virulent strain (Killar & Eisenstein, 1985). Initially the *aroA* gene was only inactivated by the insertion of the tetracycline resistant transposon Tn10 but, in order to limit reversions by clean excision, deletion mutants were constructed (Hoiseth & Stocker, 1981). A deletion mutant of *aroA* was constructed in the mouse virulent *S. typhimurium* strain SL 1344 and designated SL 3261. This strain has been used to evaluate immune responses in mice.

For additional safety from reversions to virulence, it is possible to add a second or third mutation in the aromatic amino acid pathway. *aroC* (Dougan *et al.*, 1988) and *aroD* (Miller *et al.*, 1989) mutations have been tried alone and in combination with an *aroA* deletion. The *aroC* and *aroD* mutations seem to have the same level of attenuation as the *aroA* deletion yet are still immunogenic (Miller *et al.*, 1989), thus when two *aro* deletions are combined in one organism the attenuating effect is not additive. The *aroA*, *aroC* and *aroD* genes are at 19, 47 and 36 mins respectively on the *S. typhimurium* chromosome (Sanderson & Hurley, 1987). Thus construction of *Salmonella* strains with multiple deletions in the aromatic amino acid pathway would not have an additional effect on attenuation but would contribute a greater degree of safety (Dougan *et al.*, 1988).

Aro mutants of several *Salmonella* strains have been shown to be effective oral vaccines in different species including mice (Killar & Eisenstein, 1985), calves (Smith *et al.*, 1984), sheep (Mukkur *et al.*, 1987), chickens (Cooper *et al.*, 1990) and preliminary data is available for humans (Hone *et al.*, 1992).

1.3.3 Immunological Response to Live Attenuated *Salmonella* Vaccines

Live attenuated strains of *Salmonella* have been given to animals by many different routes such as IV, IP, IM, SC and oral. The oral route has produced the most wide ranging immunological responses, presumably because the other methods (except possibly IP) may bypass the gut associated lymphoid tissue (GALT). Delivery of an antigen in *S. typhimurium* via the GALT elicits generalized secretory, humoral and cellular immune responses (Curtiss & Kelly, 1987). Different routes of administration can stimulate different arms of the immune system (Mukkur *et al.*, 1987; Wu *et al.*, 1989). IM vaccination with an attenuated *S. dublin* expressing an epitope of the Hepatitis B surface antigen was compared to oral

vaccination. With the same number of viable attenuated bacteria, IM vaccination induced higher serum antibody levels (Wu *et al.*, 1989). This may be due to the large percentage of an oral dose that is excreted but it is known that oral antigen presentation may suppress the systemic response (Wu *et al.*, 1989). However, effective and protective mucosal and cellular immunity, without detectable serum antibody, has been found in mice vaccinated orally with attenuated *S. typhimurium* expressing a malaria antigen (Sadoff *et al.*, 1988). The development of the full immune response appears to require presentation of the antigen to the GALT and then limited replication in the RES. However, persistence in the RES is not in itself enough to establish immunity as *purA* strains persist for several weeks but fail to induce significant protective immunity (O'Callaghan *et al.*, 1988).

It has been known for some time that clinical enteric infection with *S. typhimurium* in pregnant women can induce high colostrum levels of specific IgA (Allardyce *et al.*, 1974). The mechanism for this generalised secretion of mucosal IgA following stimulation at one mucosal surface has now been extensively studied. Delivery of antigen to the GALT (or bronchial associated lymphoid tissue - BALT) stimulates a generalized mucosal response involving the bronchus, breast, genitourinary tract, lacrimal glands and bowel - the "Common Mucosal Immune System" (Bienenstock *et al.*, 1978). Oral administration of attenuated *S. typhimurium* expressing the M protein of *Streptococcus pyogenes* invokes specific salivary antibodies and can protect against intranasal challenge with either wild type *S. typhimurium* or virulent *Streptococcus pyogenes* (Poirier *et al.*, 1988).

Serum responses are generally good, but vary as a consequence of the route of administration (Mukkur *et al.*, 1987) and probably require more antigen to be present than is needed for purely T-cell responses. Antibody also has a role in antibody-dependent cellular cytotoxicity which has been proposed as important in protection against *Salmonella* infection (Tagliabue *et al.*, 1986). Serum responses can be absent even when protection is demonstrated (Aggarwal *et al.*, 1991; Strugnell *et al.*, 1992).

Cellular immune responses have been detected after *Salmonella* vaccination (Killar & Eisenstein, 1985; Stocker, 1988) of both a specific and non-specific nature. Protection, consistent with non-specific activation of macrophages, was shown to begin as early as 3 days following oral vaccination and specific immunity to last for at least 7 months (Killar & Eisenstein, 1985). $CD4^+$ virus-specific T-cells (but not $CD8^+$ T-cells) have been induced by vaccination with *S. typhimurium aroA*$^-$ SL 3261 expressing the nucleoprotein of Influenza

virus (Tite *et al.*, 1990). Yang *et al.* (1990) showed that T-helper subsets can be preferentially stimulated by oral vaccination with *aroA S. typhimurium* expressing a Leishmanial surface protein. $CD8^+$ cytotoxic T-cells have been identified following vaccination with a *Salmonella* vaccine expressing the circumsporozoite antigen of *Plasmodium berghei* (Sadoff *et al.*, 1988; Aggarwal *et al.*, 1991) and the CS of *Plasmodium yoelii* (Flynn *et al.*, 1990) although the mechanism by which the antigen was able to become Class I MHC restricted is only now being elucidated (Pfeifer *et al.*, 1993). Oral or intravenous vaccination with a *aro S. typhimurium* strain expressing the P.69 antigen of *Bordetella pertussis* showed significant protection against an aerosol challenge with virulent *B. pertussis* without antibody being detected (Strugnell *et al.*, 1992). The reasons why different antigens give rise to different types of immune responses is unknown but may be due to variations in cytokine production and antigen processing (Strugnell *et al.*, 1992).

1.3.4 Expression of Heterologous Antigens

It was perceived that attenuated *Salmonella* had potential as expression vectors for antigens from other pathogens (Formal *et al.*, 1981). Antigens which are presented to the GALT and RES can stimulate generalized secretory, humoral and cellular immune mechanisms. This oral vaccination system offers the opportunity to deliver appropriate antigens by an effective route and to stimulate protective immunity. Side effects should be minimal and oral vaccination would be cost effective (Clements, 1987). Many heterologous antigens have been expressed in attenuated strains of *Salmonella* and those of bacterial origin are shown in Table 1.3, antigens of viral and eukaryotic origin are shown in Table 1.4. The expression of biologically active human Interleukin-1β from *S. typhimurium* has been shown and offers a means to deliver therapeutic proteins *in vivo* (Carrier *et al.*, 1992). Several recent reviews have been published describing these recombinant organisms (Curtiss, 1990; Hormaeche, 1991; Cardenas & Clements, 1992). Protective immunity induced by heterologous antigens expressed from attenuated *Salmonella* has been demonstrated for the following antigens: *Shigella flexneri* antigens in mice (Baron *et al.*, 1987) and *Shigella sonnei* in humans (Black *et al.*, 1987), *Streptococcus pyogenes* M protein (Poirier *et al.*, 1988), Malarial sporozoites (Sadoff *et al.*, 1988), tetanus toxin (Fairweather *et al.*, 1990), Influenza virus (Tite *et al.*, 1990) and P.69 antigen of *Bordetella pertussis* (Strugnell *et al.*, 1992). Partial protection was demonstrated with expression of Influenza virus

Antigen	Comments and references
E. coli antigens	CFA I fimbriae and heat-stable toxin expressed in a *galE* strain of *S. typhi*. Not tested in animals (Yamamoto *et al.*, 1985).
E. coli LT-B	B subunit of heat-labile enterotoxin expressed in aromatic-dependent *S. dublin* producing neutralising antibody (Clements *et al.*, 1986). LT-B has also been expressed in an *aroA* *S. typhimurium* strain (Maskell *et al.*, 1987) and a *galE S. typhi* (Clements & El-Morshidy, 1984).
Shigella antigens	*Shigella flexneri* 2a type and group antigens into *galE S. typhi*. Mice protected against challenge with virulent *S. flexneri*. (Baron *et al.*, 1987). *Shigella sonnei* form I antigen plasmid was expressed in *S. typhi* and protected mice (Formal *et al.*, 1981) and man (Black *et al.*, 1987). A LamB-Shiga toxin fusion induced an antibody response (Su *et al.*, 1992)
Streptococcus mutans antigens	As anti-caries vaccine the surface protein antigen SpaA was expressed in the cAMP mutants of *S. typhimurium* (Curtiss *et al.*, 1988). A glucosyltransferase GtfA has also been expressed (Cardenas & Clements, 1992)
M protein	*Strep. pyogenes* M protein expressed in *aro S. typhimurium*. The M protein was not detectable on the surface of the vaccine strain yet produced a strong protective immune response. The mice were also protected against intranasal challenge after oral vaccination and specific IgA was found in the saliva (Poirier *et al.*, 1988).
E. coli K88	K88 adherence antigen expressed in *galE* and *aroA S. typhimurium*. Anti-K88 antibodies were detected (Dougan *et al.*, 1986).
β-galactosidase	β-galactosidase was expressed intracellularly in *aroA S. typhimurium*. It elicited both humoral and cellular immunity in mice. (Brown *et al.*, 1987).
Brucella abortus 31 kDa protein	Oral vaccination with *Δcya Δcrp S. typhimurium* expressing this protein induced mucosal and systemic antibody responses, but not delayed type hypersensitivity (Stabel *et al.*, 1991).
Flagella expression system for epitopes.	Antigen epitopes tested include Cholera toxin (Salete *et al.*, 1989), and *Strep. pyogenes* M protein epitope (Stocker, 1990). Antibodies against the epitopes are produced.
Vibrio cholerae antigens	*S. typhi* Ty21a expressing O-antigens produced antibody responses and partial protection in humans (Tacket *et al.*, 1990).
Mycobacterium leprae antigens	*Mycobacterium leprae* protein antigens have been expressed in *Δcya Δcrp Δasd S. typhimurium* (Clark-Curtiss *et al.*, 1990).
C-fragment of tetanus toxin	Oral vaccination protects mice against toxin challenge (Fairweather *et al.*, 1990; Strugnell *et al.*, 1990)
Treponema pallidum antigen	Lipoprotein expressed from chromosomally integrated gene and expression detected by Western blotting (Strugnell *et al.*, 1990)
Francisella tularensis protein	A 17 kDa lipoprotein expressed in *Δcrp Δcya S. typhimurium* induced antibody and reduced bacterial counts of a challenge strain (Sjostedt *et al.*, 1992).
Bordetella pertussis antigens	P.69 antigen expressed on *S. typhimurium* surface induced protective immunity without detection of P.69 antibody (Strugnell *et al.*, 1992). Pertussis toxin S1 subunit was expressed in *S. typhimurium* SL 3261 and induced serum and mucosal antibody (Walker *et al.*, 1992). Filamentous Hemagglutinin antigen expressed in *aroA S. dublin* stimulated IgG, IgM and IgA antibody in mice (Molina & Parker, 1990).
Neisseria meningitidis OMP	Outer Membrane Protein expressed in *S. typhimurium* SL 3261 in outer membrane induced an antibody response but not shown to be protective (Tarkka *et al.*, 1989).
Chlamydia trachomatis MOMP	Epitopes of the major outer membrane protein expressed as LamB fusions which were located in the outer-membrane. Induced a weak serum IgG response (Hayes *et al.*, 1991).

Table 1.3 Bacterial heterologous antigens which have been expressed in *Salmonella*

Antigen	Comments and references
Dengue virus envelope antigen	76% of E gene stably expressed but problems with animal model (Cohen *et al.*, 1990)
Rotavirus glycoprotein	VP7-β-gal fusion expressed in *S. typhimurium* SL 3261 induced anti-β-gal antibodies but not anti-VP7 (Salas-Vidal *et al.*, 1990)
Influenza virus nucleoprotein	Expressed in *S. typhimurium* SL 3261 (Tite *et al.*, 1988) and used to induce protective immunity in mice (Tite *et al.*, 1990)
Herpes simplex virus glycoprotein	Glycoprotein D expressed in *S. typhimurium aroA* for use in Guinea pig model (Bowen *et al.*, 1990).
Hepatitis B virus surface and nucleocapsid antigens	Expressed as fusions with *E. coli* LT-B or as pre-S2 B-cell epitopes fused to the nucleocapsid protein. Elicit high-titre antibody responses (Schodel & Will, 1989; Schodel *et al.*, 1991)
HIV-1 envelope protein	Using a two plasmid system and fusing an epitope of HIV-1 env gene to gene 10 of phage T7. Animal studies reported in progress (Tijhaar *et al.*, 1992)
SIV P27 *gag*	Expression varies between different *Salmonella* strains (Strahan *et al.*, 1992)
OmpA expression system	Influenza virus haemagglutinin expressed as fusion with OmpA partially protected mice against virus challenge (Pistor & Hobom, 1990). *Plasmodium falciparum* antigens (730 amino acids) expressed as OmpA protein fusions are located on the surface of *S. typhimurium* (Schorr *et al.*, 1991).
Flagella expression system for epitopes	Epitopes (approximately 20 amino acids) can be expressed in functional flagella and include ones from the Hepatitis B surface protein, the HIV gp160 surface protein and the murine cytomegalovirus immediate-early protein, pp89 (Stocker, 1990).
Circumsporozoite proteins (CS)	*Plasmodium berghei* CS expressed by *S. typhimurium* induced cell-mediated protection to sporozoites in the absence of antibody (Sadoff *et al.*, 1988; Aggarwal *et al.*, 1991). The *Plasmodium yoelii* CS gene was chromosomally integrated and induced cytotoxic T-cells (Flynn *et al.*, 1990).
Trypanosoma cruzi neuraminidase	*Salmonella* expressing the protein elicited a strong antibody response to the antigen (Flynn *et al.*, 1990).
Leishmanial surface protein	Preferentially induced Th_1 cells and antibody and partially protected mice against challenge (Yang *et al.*, 1990).
Schistosome surface antigen	Expressed in *S. typhimurium* but no further reports of use (Taylor *et al.*, 1988).
Human Respiratory Syncytial Virus Glycoprotein	Expressed in *S. typhimurium* and recombinant protein shown to induce neutralising antibodies when given to Cotton rats (Martin-Gallardo *et al.*, 1993)

Table 1.4 Antigens of Viral and Eukaryotic origin which have been expressed in *Salmonella*

haemagglutinin (Pistor & Hobom, 1990), a Leishmanial surface protein (Yang *et al.*, 1990) and a membrane protein of *Francisella tularensis* (Sjostedt *et al.*, 1992).

The cellular location of the antigen can affect the immune response. Although it was felt that surface expression would be desirable, many workers have found that immune responses are generated by cytoplasmic antigens such as β-galactosidase (Brown *et al.*, 1987). It is difficult to design controlled studies to evaluate the immune responses to cytoplasmic and surface antigens as the immunological character of the antigen will alter depending on its cellular processing and location. Various systems are available to express antigens or epitopes on the surface of the bacteria (Stocker, 1990) but surface expression is not a prerequisite for an immune response. Probably the main problem found has been to stably express the antigens in sufficient quantity (Hormaeche, 1991). Potential ways to overcome these problems have been reviewed by Charles & Dougan (1990). Thus it can be seen that a range of antigens have been successfully expressed in *Salmonella* and protective responses have been elicited in some cases.

1.4 Aim of the project

Anthrax is an important disease for which an adequate vaccine does not exist. A new vaccine is needed and a vaccine vector expressing PA offers many possible advantages. Rationally attenuated *Salmonella* were the most suitable vaccine vector for the expression of PA for the following reasons. A bacterial vector might be expected to express a bacterial protein to higher levels than might a eukaryotic system, in terms of the regulatory regions of the gene and the codon usage, although there are differences between *Bacillus* and *Salmonella* in these areas. *Salmonella* are orally active yet can generate a range of immune responses. It is known that anti-PA antibody alone is not correlated with protection against *B. anthracis* challenge and so other immune responses need to be generated (probably CMI). Clinical trials are underway to evaluate the use of *aro S. typhi* as human typhoid vaccines, and if this becomes approved it should facilitate its use as a vaccine vector. Bacterial vaccines are cheap to produce and can be lyophilised to ease and prolong their storage. Oral vaccines can be administered by non-medically trained personnel, without the need for sterile equipment. *Salmonella* are related to *E. coli* and this will facilitate genetic manipulation techniques.

The *Bacillus* gene for PA was available on a plasmid, pPA26. Initial reports have suggested that PA was poorly expressed from plasmid pPA26 in *E. coli* (Vodkin & Leppla, 1983). It was decided to address these problems in a rational manner in order to produce stable high level expression of PA in *Salmonella*. The constructs that were made are shown in a fold-out page at Annex A. The most suitable construct developed would be expressed in an *aroA S. typhimurium* strain designated SL 3261 and tested in animals for the induction of protective immune response against *B. anthracis* challenge. This would demonstrate the suitability of this approach in developing a new vaccine.

Therefore, the aim of this project is to produce an attenuated *S. typhimurium* strain which stably expresses PA and induces protective immune responses against *B. anthracis* when given to mice.

CHAPTER 2

Materials and Methods

2.1 Materials

Inorganic chemicals and solvents were obtained mainly from Sigma Chemical Co. Ltd (Poole, Dorset), BDH Ltd. (Poole, Dorset) or Boehringer Mannheim UK Ltd. (Lewes, East Sussex).

Tryptone, yeast extract and "Bacto" agar were obtained from Difco Laboratories (West Mosely, Surrey) or Oxoid Ltd. (Basingstoke, Hants).

The majority of biochemicals were from Sigma, Boehringer or Bio-Rad (Hemel Hempstead, Herts).

Anti-PA mouse monoclonal antibodies (designated 14C7, 2D3 and 3B6) were produced by Dr D Williamson, CBDE, from cell lines provided by USAMRIID (Little *et al.*, 1988). The horse polyclonal antisera was raised against the spore vaccine at CBDE. Enzyme labelled antibodies were from Sigma or Bio-Rad.

Restriction endonucleases and most other DNA modifying enzymes were from Boehringer or CP Laboratories (Bishop's Stortford, Herts).

Plasmids and phagemids were from Pharmacia LKB Biotechnology Ltd. (Milton Keynes, Bucks), Stratagene Ltd. (Cambridge) or Boehringer.

Unless otherwise stated, the water used was distilled water.

2.1.1 Strains

The strains used in this study are described in Table 2.1

2.1.2 Plasmids

The plasmids and phagemids used in this study are described in Table 2.2

2.2 General Methods

2.2.1 Centrifugation

Small samples were centrifuged in a bench top Hermle Z-230M microfuge (Anderman and Co. Ltd., Kingston-upon-Thames, Surrey). Larger volumes were centrifuged in either a Sorval RT-6000 or a Beckman J2-20 centrifuge (Beckman Instruments, Inc., High Wycombe, Bucks) using a JA-10 or JA-20 rotor. Ultracentrifugation was performed in a Beckman 70.1Ti rotor in an L8-M centrifuge.

Escherichia coli				
Strain	Relevant Genotype	Comments	Source	Reference
HB 101	supE44 hsdS20($r_B^-m_B^-$) recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1	Transformable strain. Host for plasmids with lacI and for cell fractionation experiments	Laboratory stocks	Bolivar & Backman (1979)
JM 101	supE thi Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔm15]	Host for pUC and pBluescript plasmids	Stratagene Ltd., Cambridge	Messing (1979)
JM 109	recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB$^+$ lacI^q lacZΔm15]	Recombination deficient derivative. Host for pUC and pBluescript	Stratagene Ltd.	Yanisch-Perron *et al.* (1985)
Salmonella typhimurium				
Strain	Relevant Genotype	Comments	Source	Reference
LB 5010	r m$^+$ galE P1^s	Used to modify *E. coli* plasmid DNA for electroporation into SL 3261	Prof G Dougan, Wellcome Labs, Beckenham	Bullas & Ryu (1983)
SL 1344	hisG46	WRAY lineage, calf isolated, mouse virulent	Dr BAD Stocker, Stanford, USA	Smith *et al.* (1984)
SL 3261	aroA DEL406, hisG46	Derived from SL 1344; a vaccine strain	Dr BAD Stocker	Hoiseth & Stocker (1981)
Bacillus anthracis				
Vollum 1B	Toxigenic and encapsulated	Isolated from a cow, Oxford , 1944. Virulent. NCTC 10340.	CBDE	Turnbull *et al.*, (1992)

Table 2.1 Bacterial strains used in this study

Vectors

Designation	Marker	Promoter	Comments	Reference
pUC18/19	Amp^r	*lac*	*lacZ'* cloning vector	Yanisch-Perron *et al.* (1985)
pBR322	Amp^r, Tet^r		cloning vector	Bolivar et al (1977)
pBluescript II KS+	Amp^r	*lac*	*lacZ'* phagemid	Alting-Mees & Short (1989)
pTrc99A	Amp^r	*trc*	high expression plasmid	Pharmacia LKB Ltd., Milton Keynes, Bucks

Recombinant Plasmids

Designation	Vector	Comments	Reference
pPA26	pBR322	Plasmid with *B. anthracis* DNA containing the PA gene	Vodkin & Leppla (1983)
pFS2.2	pUC19	Plasmid expressing LT-B from *lac* promoter. Used in the construction of pSIG13	Schodel & Will (1989)
pKS492	pUC18	Contains *cer* locus	Prof. D Sherratt, Univ. of Glasgow
pCB1	pBluescript	Recombinant expressing full-length PA	This study
pORF1	pBluescript	Recombinant expressing full-length PA	This study
pORF1c	pBluescript	Recombinant as for pORF1 with *cer* locus	This study
pVET4m	pBluescript	Recombinant expressing 63 kDa PA, *lac*	This study
pVET4n	pBluescript	Recombinant expressing 63 kDa PA, *lac*	This study
pNIK1n	pTrc99A	Recombinant expressing 63 kDa PA, *trc*	This study
pNIK1m	pTrc99A	Recombinant expressing 63 kDa PA, *trc*	This study
pSIG13	pUC19	Recombinant expressing 63 kDa PA, *lac*	This study
pBPA9	pBluescript	Recombinant phagemid containing the sequence for the PA-63 with an unmodified C-terminus. Used in construction of pNIK1n.	This study

Table 2.2. Plasmids and Phagemids used in this study

2.2.2 Sterilisation

Heat stable solutions were sterilised in an autoclave on a liquid cycle. Other solutions were sterilized by passing through a 0.22μm pore size membrane. Pipette tips and instruments were sterilized in an autoclave on a steam cycle.

2.3 Microbiological Methods

2.3.1 Preparation of Culture Media

2.3.1.1 Routine Media

LB-medium (L-broth or L-agar)

L-broth and L-agar were used for all routine cultures of both *E. coli* and *S. typhimurium*.

L-Medium

Tryptone	10 g
Yeast extract	10 g
NaCl	5 g

Make to one litre and adjust pH to 7.0 with 5N NaOH; for L-agar add 15 g Bacto-agar before autoclaving.

Glucose Minimal Agar

Glucose minimal agar (GMA) was used for maintaining the F' episome in certain strains of *E. coli*.

GMA

10x M9 salts	100 ml
20% Glucose	20 ml
Minimal agar	15 g

Make to 1 litre and before use supplement with 1mM $MgSO_4$, 0.1mM $CaCl_2$ and 1mM thiamine.

10x M9 Salts

Na_2HPO_4-$7h_2O$	128 g
KH_2PO_4	30 g
NaCl	5 g

NH_4Cl 10 g

Dissolve in water to 1 litre. Divide into aliquots and autoclave 15 mins at 15 lb/sq inch liquid cycle.

2xTY Medium

This medium was used in the preparation of ssDNA from phagemids.

Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Add to 900 ml water, adjust to pH 7.0 with 5N NaOH and make to 1 litre.

SOC Medium

This medium was used to recover bacteria after electroporation.

Tryptone	20 g
Yeast extract	5 g
NaCl	0.5 g

Add to 950 ml water and shake to dissolve. Add 10 ml of 250mM KCl and adjust to pH 7.0 with 5N NaOH. Make to 1000 ml and autoclave. Before use add 20 ml of a filter sterilized 1M glucose solution and 5 ml of a filter sterilized 2M $MgCl_2$ solution.

Minimal Media for *aro* mutants

aro and *his* mutants were identified by growing on minimal media supplemented as required with aromix and/or histidine.

Minimal medium

170 ml 2% noble agar (Difco)

20 ml 10x M9 Salts

10 ml 20% Glucose

2 ml Ca/Mg Salts

1% v/v Aromix (if required)

1% v/v Histidine -4mg/ml stock (if required)

Ca/Mg Salts

10mM $CaCl_2$

100mM $MgSO_4$

Make separately, autoclave, mix in 1:1 ratio before use.

Aromix

Phenylalanine	4mg/ml
Tryptophan	4mg/ml
Para-aminobenzoic acid	1mg/ml
Dihydroxybenzoic acid	1mg/ml

Filter sterile. Make Tyrosine (4mg/ml) separately and add to medium at 1% v/v before use.

Phosphate-buffered saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

Make to 800 ml and adjust pH to 7.4 with HCl, make to 1 litre and autoclave.

2.3.1.2 Media supplements

Media was supplemented as required with:

	Stock solution	Amount /100 ml media	Working concentration
Ampicillin	25 mg/ml	0.4 ml	100 μg/ml
Chloramphenicol	34 mg/ml in ethanol	0.5 ml	170 μg/ml
Kanamycin	10 mg/ml	100 μl	10 μg/ml
Thiamine	0.1%	1 ml	0.001%
IPTG	1M	100 μl	1mM
X-gal	20 mg/ml in N,N-dimethyl formamide	1.6 ml	320 μg/ml

2.3.1.3 Culture conditions

Cultures were grown at 37°C under aerobic conditions. Small volumes were grown in 20 ml Universal tubes and larger volumes in 250 ml flasks. Cultures were routinely incubated in an orbital shaker except for animal inoculation cultures which were grown statically.

2.3.2 Plate counts

Plate counts were used to determine the number of viable bacteria in cultures and tissue homogenates and to assess the ratio of antibiotic resistant to sensitive cells. Two methods were used.

Manual counting. Serial 10 fold dilutions in PBS were made and 100 μl of appropriate dilutions plated out on L-agar plates with antibiotic supplementation if required. For cultures the Abs_{600} was used to estimate the cell density; it was found that for *S. typhimurium* an Abs_{600} of 1 equated to $2x10^9$ bacteria. For tissue homogenates, a wider range of dilutions were plated out as an estimate of the bacterial count was not available. The plates were incubated overnight at 37°C. Colonies on the plates were counted manually.

Automated method. From an estimate of the cell density, the culture was diluted with ice-cold PBS to produce approximately a 10^4 to 10^6 cfu/ml culture. 50 μl of this dilution were plated out using a Spiral Plater Model D (Spiral Systems Inc., Cincinnati, USA). After overnight incubation at 37°C, the plates were placed in a Laser Colony Counter Model 500A (Spiral System Instruments Inc., Bethesda, USA) and the cfu/ml count determined from the distribution of colonies along the spiral track using Bacterial Enumeration Software BEN CASBA II (Spiral System Instruments Inc., Bethesda, USA).

2.4 DNA Methods

2.4.1 Phenol extraction and ethanol precipitation of DNA

DNA in aqueous solution was extracted with an equal volume of Tris-buffered phenol/chloroform followed by an equal volume of chloroform. DNA was precipitated from aqueous solution by adjusting the salt concentration to either 0.3M sodium acetate (using 3M sodium acetate pH 5.5) or 2M ammonium acetate (using 10M ammonium acetate) and adding 2 volumes of absolute ethanol and storing at -20°C for 10 mins to overnight and centrifuging in a microfuge for 10 mins. When necessary, salt was removed from the pellet

by washing in 70% ethanol. Pellets were air dried and resuspended in an appropriate volume of distilled water or TE buffer.

Tris buffered phenol/chloroform

100 ml phenol (melted at 65°C)

96 ml chloroform

4 ml isoamyl alcohol

200 μg 8-hydroxyquinoline

Equilibrate with 1M Tris pH 8.0, cover with 0.1M Tris pH 8.0.

2.4.2 Plasmid preparations

2.4.2.1 Small-scale plasmid preparations

The protocol used was a modification of the Birnboim & Doly (1979) alkaline lysis method as amended in Sambrook *et al.* (1989). Basically 1.5 ml of culture were pelleted and resuspended in 100 μl of Buffer A (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA). After 5 mins on ice 200 μl of Buffer B (0.2M NaoH, 1% SDS) were added and after a further 5 mins on ice 150 μl of Buffer C (2M sodium acetate) were added and the precipitate spun down after 3-5 mins. The supernatant was phenol:chloroform extracted and the DNA precipitated with ethanol.

2.4.2.2 Large-scale plasmid preparations

Two methods were used:

a. Chloramphenicol amplification followed by SDS lysis and purification by equilibrium centrifugation in CsCl-ethidium bromide gradients (Sambrook *et al.*, 1989)

b. Use of the Qiagen >plasmid<maxi kit for culture volumes of 150 ml (for high copy number vectors) as directed by the manufacturer (Qiagen, FRG)

2.4.3 Restriction Digests

Restriction endonucleases were obtained from Boehringer Mannheim UK Ltd. and CP Laboratories and were used with the supplied buffers and in accordance with the manufacturer's directions.

2.4.4 DNA electrophoresis and fragment purification

DNA in final sample buffer was electrophoresed in horizontal agarose gels consisting of from 0.7% to 1.5% (w/v) agarose (Gibco Ltd., Uxbridge, Middlesex) in TBE containing 0.5 μg/ml ethidium bromide. Bands were visualised under UV light.

Sample Buffer (x6)

50% (v/v) Glycerol

0.25% (w/v) Bromophenol blue

0.1M EDTA

in distilled water

TBE buffer(x10)

54.5 g Tris

27.5 g Boric Acid

4.2 g Disodium EDTA

make to 500 ml with distilled water

DNA fragments were purified from gels by one of three methods:

2.4.4.1 DEAE Cellulose

The method of Dretzen *et al.* (1981) as modified by Sambrook *et al.* (1989) was used. DEAE membranes (NA45 membranes, Schleicher-Scheull) were activated by soaking for 5 mins in 10mM EDTA, pH 8.0 followed by 5 mins in 0.5N NaOH, washed six times in water and stored in water at 4°C. The appropriate band was run onto the membrane, the membrane was washed in 10 ml of low salt buffer (50mM Tris pH 8, 0.15M NaCl and 10mM EDTA) and placed into an eppendorf tube. The membrane was covered in high salt buffer (HSB - 50mM Tris pH 8, 1M NaCl and 10mM EDTA) and incubated at 65°C for 30 mins. The HSB was removed and a second aliquot added for a 15 min incubation. Both aliquots were combined, the DNA purified by phenol extraction and ethanol precipitated in the presence of 2M ammonium acetate. The DNA was resuspended in distilled water.

2.4.4.2 Qiaex

Qiaex silica gel (Qiagen, FRG) was used according to the manufacturer's instructions and with the buffers provided in the Qiaex >gel extraction< kit. This method of fragment purification was found to give the best results.

2.4.4.3 Agarase

Agarase was purchased from Boehringer Mannheim and used according to the manufacturer's instructions and the modified method of Maule (1992). The DNA samples were electrophoresed on a 0.8% LMP agarose gel. The band was excised and an equal volume of TAE buffer added and the gel melted at 65°C for 10 mins. The solution was cooled to 45°C and 1 Unit of agarase/100 μl of solution added. The mixture was incubated at 45°C for 60 mins and then cooled and 1/10 volume of 3M sodium acetate added and the tube stored on ice for 10 mins. The undigested agarose was pelleted and the supernatant carefully removed. 3 volumes of ethanol was added and the tube held at -20°C for 60 mins before the DNA was pelleted in a microfuge. The pellet was dried and resuspended in an appropriate volume of water or TE buffer.

2.4.5 Ligation reactions

Bacteriophage T_4 DNA Ligase was purchased from Boehringer Mannheim and ligations were performed in the manufacturer's buffer at 15°C overnight for sticky-ended ligations. Blunt-ended ligations were performed according to the method of Sambrook *et al.* (1989) using 0.5mM ATP, 15% PEG 8000 and 0.5 Weiss Units of T_4 Ligase per 10 μl reaction mix.

2.4.5.1 CIP treatment

Calf Intestinal Phosphatase (CIP) was used to dephosphorylate vector DNA to reduce recircularization of vector DNA without incorporation of an insert. Purified DNA was treated in CIP dephosphorylation buffer with CIP at a concentration of 1 unit/100 pmoles of DNA. Restricted DNA was CIP treated in the restriction buffer by adding $ZnCl_2$ to a concentration of 1mM and then the appropriate amount of CIP. The reaction mix was incubated at 37°C for 30 mins. EDTA was added to 5mM and the CIP inactivated at 75°C for 10 mins.

CIP dephosphorylation buffer (10x)

10mM $ZnCl_2$

10mM $MgCl_2$

100mM Tris (pH 8.3)

2.4.6 Oligonucleotide synthesis

Oligonucleotides were used as sequencing primers, PCR primers and for the construction of DNA linkers in plasmid construction. They were synthesised by Dr. D Anderson (CBDE, Porton Down) on a 200A DNA synthesizer (Beckman) using cyanoethyl-phosphoramidite chemistry. Oligonucleotides were supplied in the deprotected form, cleaved from the phosphoramidite column and dried from the final ammonium solution. Routinely primers were dissolved in water, ethanol precipitated, resuspended in distilled water and quantified by spectrophotometric methods.

2.4.7 Sequencing Methods

2.4.7.1 Template preparation

Four types of template were used in sequencing:

a. Phagemid ssDNA. The pBluescript II vectors can be rescued as single stranded DNA as they contain an M13-related f1, filamentous phage, intergenic region. The phagemid must be present in an F' bearing plasmid and so for ssDNA preparation the plasmids were electroplated into *E. coli* JM 109 which had been maintained on a glucose minimal agar plate. The recombinant bacteria were grown on an L-agar plate supplemented with ampicillin and a colony was inoculated into 2 ml of 2xTY broth supplemented with ampicillin and thiamine. This was grown to an Abs_{600} of 0.5 to 0.8. The culture was infected with the helper phage M13K07 at a multiplicity of infection of 10 phage/bacteria and incubated for 1 hour. 100 ml of 2xTY were supplemented with ampicillin and kanamycin and inoculated with the phage infected culture and incubated overnight. The cells were pelleted and the phage precipitated from the supernatant with 20% PEG8000 in 2.5M NaCl at a ratio of 1 ml PEG to 5 ml of supernatant. The phage was pelleted by centrifugation and resuspended in 5 ml of water. The phage coat was removed by phenol/chloroform extraction and the ssDNA precipitated with ethanol and resuspended in a final volume of 200μl of water.

b. Dynabead ssDNA. The Dynabead Template Preparation kit (Dynal (UK) Ltd. Wirral) was used to prepare ssDNA according to the manufacturer's instructions except for the following modifications. The non-biotinylated strand was removed with 100 μl of 0.15M NaOH and the Dynabeads were resuspended in 15 μl of water so that they could be used for 2 sequencing reactions (7 μl per reaction). In some cases the PCR primers used were biotinylated-T3 and T7 primers (see Table 2.4) which correspond to these regions in pBluescript. For long inserts, higher primer concentrations were used in the PCR reaction than is recommended. As the unused primers would block streptavidin sites on the beads. the PCR product was precipitated with Ammonium acetate and ethanol before binding to the beads.

c. Denatured dsDNA. Purified plasmid DNA or PCR product was sequenced following alkaline denaturation as described in the 'Sequenase Version 2' kit (United States Biochemical Corp., Cleveland, Ohio). 3-5 μg of plasmid DNA were denatured in 0.2M NaOH, 0.2mM EDTA for 30 min at 37°C. 0.1 volumes of 3M sodium acetate were added and the DNA precipitated with 3 volumes of ethanol. The pellet was resuspended in 7 μl of water and sequenced as for ssDNA.

d. dsDNA. Cycle sequencing can function with a dsDNA template as the template is denatured during the heating cycles. Plasmid DNA was purified using Qiagen columns.

2.4.7.2 Sequencing reactions

Except for cycle sequencing, DNA sequencing reactions were performed using the dideoxy chain termination method of Sanger *et al.* (1977) using cloned T7 DNA polymerase supplied in a sequencing kit form (Sequenase Version 2, United States Biochemical Corp.). Sequencing primers were synthesised at CBDE. Primers used are listed in Table 2.3. The manufacturer's instructions were followed with the labelling mix diluted 1:3 in water. Routinely labelling was carried out for 5 mins at room temperature and termination reactions were incubated at 37°C for 5 mins. Before the stop solution was added a "Taq chase" was usually used. dITPs and dI labelling mix were used to resolve areas of CG compressions according to the manufacturer's instructions.

Name	5' to 3' sequence	Comments
PAS1	ATA GTG TTT TCT TTA GTA	PA sequencing primer (4000 in pPA26 insert)
PAS2	TGA TAA TAT TTT TCT TAT	PA sequencing primer (3800)
PAS3	TGT TAG TTG CGT TTA ATT	PA sequencing primer (3600)
PAS4	GCC GCT ATC CGC CTT TCT	PA sequencing primer (3400)
PAS5	AAG AAA TTG ATT GTA ATT C	PA sequencing primer (3200)
PAS6	TTG GAG CCG TCC CAG TAT	PA sequencing primer (3000)
PAS7	GCA TTT CCA TGT ACT TCA	PA sequencing primer (2800)
PAS8	TGT AAC CTT TTC GAA ATC ACT GTA	PA sequencing primer (2600)
PAS9	TAG GTC CAG CAC TTG TAC	PA sequencing primer (2400)
PAS10	CTA AAA AAG GCT ATG AGA	PA sequencing primer (4073)
PAS11	CTT CTC TCA TCC GCC AAA	pTrc99A (350) PCS sequencing into insert
PAS12	CTG ATT TTG TTA GAA TTA	PA sequencing primer (2200)
PAS13	TGT AGT AGA AGA GGT AAC	PA sequencing primer (2000)
PAS14	GTT TAA CTT CTG CCT GAA	PA sequencing primer (1900)
PAS7R	ACA TGT AGA TAT GGA GAA	PA sequencing primer for complimentary strand (2680)
Universal Primer	GTA AAA CGA CGG CCA GT	M13 -20 Primer
SK Primer	TCT AGA ACT AGT GGA TC	pBluescript sequencing primer

Table 2.3 Sequencing primers used in this study. Sequences are shown 5' to 3'. The figure in brackets in the 'Comments' column refers to the site that the primer binds at within the gene or plasmid.

Cycle sequencing was performed using a CircumVent kit (New England Biolabs, Bishop's Stortford) according to the manufacturer's instructions. 200 ng of dsDNA template were used and approximately 10 pmol of primer. The manufacturer recommends 1.2 pmol of primer, but excess primer was found to improve the sequence ladder. ^{35}SdATP was incorporated into the sequence products by cycling the reactions on a PCR machine (PREM III, LEP Scientific, Milton Keynes) for 30 cycles of 95°C for 30 secs, 55°C for 30 secs, 72°C for 30 secs.

2.4.7.3 "Taq chase"

To help resolve areas of G/C compressions and sequence over other regions containing secondary structure during non-cycle sequencing, a "Taq chase" step was added to the standard method. Taq polymerase enzyme was diluted in the manufacturer's buffer to give a concentration of 1 unit/μl. Before the addition of stop solution, 1 μl of the diluted Taq polymerase enzyme was added to each tube and the tubes incubated at 72°C for 2 mins.

2.4.7.4 Denaturing PAGE separation

Denaturing PAGE was used to separate the extension products. Sequencing plates (Sequigen sequencing apparatus, Bio-Rad) were cleaned and silanised as required. Either 21x40 cm or 38x50 cm plates were used, and gel components are listed for each size of plates. 1xTBE gel mix was made using 6% acrylamide mix and 10xTBE. The plates were sealed with 25-50 ml of gel mix after the addition of 170-350 μl of both 10% (w/v) ammonium persulfate (APS) and TEMED. Once set, the rest of the gel was poured after adding 1-2 μl/ml of gel mix of APS and TEMED to the remaining 1xTBE gel mix. A comb was inserted upside down in the top of the gel to form a well. The upper and lower buffer chambers contained 0.5xTBE and 1xTBE respectively. The gel was pre-run at 60-140W until it reached 50°C. The well was irrigated to remove urea, the comb inserted and the samples loaded in the order GATC. The gel was run at a wattage designed to maintain the temperature at 50°C. The plates were split and the gel covered in gel fixer (10% methanol 10% acetic acid) for 15 mins. The gel was transferred to filter paper, covered in Saran wrap and dried under vacuum at 80°C. After removal of the Saran wrap, the dried gel was exposed to hyperfilm-β-max (Amersham) overnight in a film cassette.

TBE (10x)

54.5 g Tris

27.5 g Boric Acid

4.2 g Disodium EDTA

make to 500 ml with distilled water

6% acrylamide gel mix

215 g Urea

75 ml 40% acrylamide/bis-acrylamide gel mix (Boehringer)

make to 450 ml, stir till dissolved and make to final volume of 475 ml. Store 4°C.

2.4.8 Polymerase Chain Reactions

The Polymerase Chain Reaction (PCR) was used for several applications. Gene fragments produced during PCR were cloned with either blunt-ends or by the incorporation of 'tails' containing restriction enzyme sites. PCR was also used to detect recombinant clones and to prepare ssDNA for sequencing.

The primers used are shown in Table 2.4. Primers were designed according to standard criteria where possible although the choice was limited where they were needed to span particular regions for cloning purposes. Primers were synthesised in-house and diluted to give a working strength solution of 1μg μl.

A reaction mix was prepared using methods to prevent contamination. The Taq polymerase manufacturer's 10x buffer was used (500mM KCl, 100mM Tris-HCl (pH 8.3) 15mM $MgCl_2$ and 0.1% gelatin). The $MgCl_2$ concentration is critical and was usually optimised. This 10x buffer gives a final molarity of 1.5mM which is at the low end of the normal range used of 1.5 to 4mM. Therefore additional $MgCl_2$ was added to identify the best concentration for a particular reaction mix. A typical reaction mix would be:

2 μl 10mM dATP

2 μl 10mM dTTP

2 μl 10mM dGTP

2 μl 10mM dCTP

10 μl 10x Buffer (supplied with the enzyme)

2 μl primer 1 (1 μg/μl for a 20mer)

Name	5' to 3' sequence	Comments
UFB	Biotin-GCT TCC GGC TCG TAT GTT GTG TG	Dynabead universal primer
UF	GCT TCC GGC TCG TAT GTT GTG TG	Dynabead universal primer
URB	Biotin-AAA GGG GGA TGT GCT GCA AGG CG	Dynabead universal primer
UR	AAA GGG GGA TGT GCT GCA AGG CG	Dynabead universal primer
T3	ATT AAC CCT CAC TAA AG	pBluescript primer
T7	AAT ACG ACT CAC TAT AG	pBluescript primer
PA 1222	AAT TCA AGT ACG GTC GCA AT	Internal PA primers
PA 1219	CTT TAA TTG TCG CGA GTG TT	Internal PA primers
ANTHPR3	ATC CGG AGC TCA GAT CTC GAA CTC AAG AAA AAA GCG A	Cloning primer for 63 kDa fragment
ANTHPR4	ATC TAG AGA GCT CCT AGG TAT CCT ATC TCA TAG CCT TTT TT	Cloning primer for 63 kDa fragment
ANPR3S	TCG AAC TCA AGA AAA AAG CGA	Complimentary region of ANTHRR3
ANPR4S	TAT CCT ATC TCA TAG CCT TTT TTA	Complimentary region of ANTHPR4
BPA3	ATC GGA GCT CCG TCG AAC TCA AGA AAA AAG CGA	Cloning primer for 63 kDa region
BPA4	ATC ACT ATC CTA TCT CAT AGC CTT TTT T	Cloning primer for 63 kDa region
PAALL5	ACA AAA GCT GGG TAC CTT TTA TAC AAA AAG GAG AAC G	Cloning primer for pORF1
PAALL3	GGT CGA CTC TAG AGG ATC CTA CAA ACA ATC TCA AAG GAT	Cloning primer for pORF1

Table 2.4 PCR Primers used in this study. Sequences are shown 5' to 3'.

2 μl primer 2 (1 μg/μl for a 20mer)

0.5 μl of Taq DNA polymerase - 5U/ml (Boehringer)

x μl of DNA template solution

y μl of H_2O to make total to 100 μl

The mixture was pipetted into a 0.6 ml eppendorf tube and placed in the heating block of a PREM III (LEP Scientific. Milton Keynes. Bucks) PCR machine. This machine automatically allows an equilibration period between temperature points to allow the tube contents to stabilize at that temperature. A typical cycle for a 1 kbp fragment would consist of:

a. 95°C for 5 mins - to completely denature the template DNA for the first cycle and inactivate any proteases or nucleases present.

b. 95°C for 30 secs - to denature the DNA
40°C for 30 secs - to anneal the primers
72°C for 1 min - to elongate the strands
repeat for 30 cycles

c. 72°C for 5 mins - to finish elongating all strands, for cloning

d. refrigerate 4°C - until ready to use product

2.4.8.1 Blunting of PCR products with Klenow

Taq polymerase is able to add a non-template-dependent base to the 3' end of extended chains. It is necessary to remove this addition in order to blunt-end clone PCR fragments. This was done using the method described by Sardelli (1991). The PCR products were gel purified and resuspended in 1x buffer or the Taq enzyme was heat inactivated (10 mins at 99°C). The $MgCl_2$ concentration was adjusted to 5mM and 1-2U Klenow enzyme added. This was incubated at room temperature for 30 mins and the Klenow inactivated at 95°C for 5 mins. The DNA was either ammonium acetate precipitated (PCR mix) or used directly (gel purified DNA).

2.4.9 Electroporation

Electroporation was used in preference to transformation of competent cells because of its ease of use and because it was also needed to move plasmids into vaccine strains, as

the competent cell transformation method selects for rough mutants. The only alternative to electroporation was P22 transduction which can be less successful than electroporation when plasmids expressing toxic proteins are transformed.

2.4.9.1 Preparation of cells

Stocks of bacteria for electroporation were prepared in advance and stored at -70°C. The cells were grown in 500 ml of L-broth to mid-log phase (Abs_{600} of 0.5-0.8). The cells were chilled on ice and harvested by centrifugation. They were washed in 500 ml of cold 10% glycerol and pelleted by centrifugation. This was repeated 3 times with the cells being resuspended in 250 ml, 50 ml and 10 ml of 10% glycerol. The cells were finally resuspended in 1 ml and divided into 50 μl aliquots and stored at -70°C.

2.4.9.2 Pulsing cells

An aliquot of cells was thawed on ice. A pulsing cuvette (Bio-Rad) was chilled and 40 μl of cells pipetted into a chilled eppendorf tube. 1-2 μl of the ligation mix or plasmid preparation were added, mixed and left on ice for 1 min. The mixture was transferred to the cuvette and placed in the pulsing chamber (Gene Pulser, Bio-Rad, Hemel Hempstead). *E. coli* were pulsed at 2.5 KV, 25 μF and 200 ohms. *S. typhimurium* were pulsed at 1.25 KV, 25 μF and 800 ohms. The pulse voltage and time constant were checked to ensure that a short circuit had not occurred. The time constant for *E. coli* should be 5 msec and for *S. typhimurium* 20 msec. After the pulse the cuvette was removed and 1 ml of SOC medium used to flush out the cells from the cuvette. The SOC was placed in a tube and incubated at 37°C for 1 hour. Aliquots of the SOC were plated out onto selective media plates and incubated overnight.

2.4.10 Computer analysis of nucleotide and amino acid data

Nucleotide and amino acid sequence were analyzed using the DNASTAR software on a IBM compatible PC. The CLONE programme was used to construct the nucleotide sequences from the vector sequence files, PA sequence file - ANTH_PA.SEQ and PCR primer sequence entered directly into the CLONE statement. The MAPSEQ programme was used to generate the complimentary sequence, identify restriction sites and translate the appropriate reading frame into corresponding amino acids.

mRNA secondary structure was predicted using the algorithm of Zuker & Stiegler (1981) which is implemented within the computer programme Genmon 4.3 (GBF, Braunschweig) on a Vax computer. The structure was predicted at a temperature of 37°C.

2.5 Protein Methods

2.5.1 Preparation of cell lysates

2.5.1.1 Whole cell - routine

Cell lysates were prepared by growing the cells in the appropriate conditions, with IPTG induction as required. For quantitative results, the Abs_{600} was measured and the cultures diluted with medium to give the same cell densities. 1.5 ml of culture were centrifuged in an eppendorf tube and the supernatant discarded. The pellet was resuspended in 100 μl of water and 100 μl of 2xLoading Buffer (2xLB - 62.5 mM Tris pH 6.8, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol) was added and boiled for 3 mins.

2.5.1.2 Whole cell - chloramphenicol amplification

A modification of the method of Little & Lowe (1991) was used to maximise plasmid copy number so as to enhance the ratio between cell proteins and recombinant proteins. This method was used to show expression of plasmid proteins which could not be detected by routine methods. 100 μl of an overnight culture were added to 30 ml of L-broth supplemented with ampicillin. The culture was grown to an Abs_{600} of 0.8 to 1.0 and chloramphenicol was added to 170 μg/ml and the culture grown overnight. The cells were pelleted and washed in L-broth. The washed cells were resuspended in 30 ml L-broth supplemented with ampicillin and incubated for 1 hour. If comparisons were to be made between cultures, the Abs_{600} was measured and the denser cultures diluted with L-broth to give the same Abs_{600} readings. A set amount of the cultures was then treated as for routine whole cell lysates.

2.5.1.3 Cell fractionation

E. coli HB 101 cells were fractionated into periplasmic and cytoplasmic fractions by a modification of the method of Sambrook *et al.* (1989). Normally the bacteria were grown

overnight in 100 ml of modified M9 media to enhance production of alkaline phosphatase, but if this media inhibited expression of the recombinant protein then L-broth and any necessary inducer was used. After measuring the Abs_{600}, 40 ml of cells were pelleted and resuspended in 1.5 ml of TES (20% sucrose (w/v), 30mM Tris (pH 8.0), 1mM EDTA (pH 8.0)), and fresh lysozyme added (1 mg/ml), and placed on ice for 10-30 mins. The spheroplasts were recovered and the supernatant stored as the periplasmic fraction. The spheroplasts were washed in TES buffer and resuspended in 1.5 ml of 100 mM Tris (pH 8.0) and ruptured by three cycles of freeze/thawing (dry ice/ethanol to 37°C) and the cell membranes and insoluble proteins pelleted to leave the soluble cytoplasmic fraction. The pellet was solubilized in 1.5 ml of 1% Triton X-100.

2.5.1.4 Recovery of periplasmic proteins

This method was used to prepare periplasmically located recombinant PA (rPA) from *E. coli* JM 109 bacteria containing phagemid pORF1. The rPA was used as an immunogen in animal experiments.

10 ml of L-broth supplemented with ampicillin were inoculated with *E. coli* JM 109 / pORF1 and grown for 6 hours, shaking at 37°C. 1 ml of this culture was inoculated into 10 flasks containing 100 ml L-broth supplemented with ampicillin. The flasks were incubated overnight at 37°C in a shaking incubator. rPA production was induced with 1mM IPTG for 4 hours. The cells were pelleted by centrifuging at 5000 rpm at 4°C for 10 minutes in a Beckman centrifuge. The pellet was resuspended in 300 ml cold 10mM Tris, pH 7.4 and repelleted. The cells were resuspended in 100 ml 10 mM Tris, 0.58M sucrose, pH 8.0 at room temperature. 2.2 ml of 2mg/ml lysozyme were added followed 15 sec later by 4.5 ml o.1M EGTA. This was incubated for 10 min at room temperature. 1.1 ml of 100mM Phenylmethylsulphonyl Fluoride (PMSF) were added and the spheroplasts pelleted by centrifugation at 10,000 rpm for 20 min at 4°C in the Beckman centrifuge. The supernatant was recovered (approx 100 ml). The protein was precipitated with 70% ammonium sulphate (47.2 gm ammonium sulphate per 100 ml) added slowly to the stirred supernatant which was maintained at 0-4°C. The precipitate was left at 4°C overnight and then centrifuged at 14,000 rpm for 60 min at 4°C. The supernatant was removed and the precipitated proteins resuspended in 5 ml cold PBS and dialysed against 4x one litre changes of PBS at 4°C. The solution was spun in an ultracentrifuge using a swing out rotor (55Ti)

at 32,000 rpm for 30 min to pellet contaminating LPS and other solid material. The supernatant was filter sterilized through a 0.22μm filter and stored in aliquots at -20°C.

2.5.2 Enzyme assays

Enzyme assays were used to show the accuracy of the cell fractionation method. *E. coli* HB 101 was used as this has the appropriate enzymes present.

2.5.2.1 Alkaline phosphatase enzyme assay

Cell fractions were assayed for the presence of alkaline phosphatase which is present in the periplasm in large amounts when the cells are cultured in low phosphate conditions. The assay is as described by Torriani (1967). Samples were diluted in 0.6M Tris pH 8.2 to give a volume of 0.9 ml. The substrate was p-nitrophenolphosphate (PNPP) at a concentration of 0.0066M (17.4 mg in 10 ml of 0.6M Tris buffer). The samples were placed in cuvettes and maintained at 28°C in a water bath. 100 μl of the substrate were added to each cuvette and the increase in absorption at 414nm, due to the formation of yellow p-nitrophenol, was monitored at several time points. Values were compared to a blank containing buffer and substrate. Enzyme units/ml of sample were calculated as:

$$\frac{\textit{Enzyme units}}{\textit{ml}} = \frac{A_{410}}{\textit{time (mins)}} X \textit{ dilution factor}$$

2.5.2.2 β-galactosidase enzyme assay

Cell fractions of HB 101, which carries the *lacZ* gene on the chromosome, were assayed for β-galactosidase activity, an enzyme that is found cytoplasmically. The assay was described by Miller (1972). The samples were diluted in Z buffer to a final volume of 0.8 ml, placed in capped cuvettes and maintained at 28°C in a water bath. The substrate was 4 mg/ml o-nitrophenyl-β-D-galactoside (ONPG) in 0.1M phosphate buffer pH 7.0 (61 ml 0.1M Na_2HPO_4 and 39 ml of 0.1M NaH_2PO_4). 0.2 ml of substrate was added to each cuvette and the increase in absorbance at 420nm monitored as this represented the formation of yellow 0-nitrophenol. The values were compared to a blank containing only buffer and substrate. The absorbance at 550nm was also monitored as this represented the light scattering due to debris and was used as a correction factor. The enzyme units /ml of sample were calculated as:

$$\frac{Enzyme\ units}{ml} = \frac{A_{420} - 1.75XA_{550}}{time\ (mins)}\ X\ dilution\ factor\ X\ 1000$$

Z Buffer

$Na_2HPO_4.7H_2O$	0.06M
$NaH_2PO_4.H_2O$	0.04M
KCl	0.01M
$MgSO_4.7H_2O$	0.001M
β-mercaptoethanol	0.05M

adjust pH to 7 with orthophosphoric acid.

2.5.3 Proteolytic cleavage of PA - Trypsin digests

Full length PA was treated with trypsin to cleave the 83 kDa protein into 63 kDa and 20 kDa fragments. These could be used as markers on Western blots. Agarose beads coated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treated insoluble trypsin (Sigma) were washed in Tris/Glycine (25mM Tris pH 8.0, 0.2M Glycine) buffer and resuspended in buffer to give 38 Units activity/ml. Freeze-dried PA was reconstituted with water to give a concentration of 1.75 mg/ml. 50 μl of this PA solution were mixed with 60 μl of the washed trypsin beads and incubated for 30 mins at room temperature. The tube was spun to pellet the beads and the supernatant removed and 50 μl of 2xLB added. This was boiled for 2 mins. For SDS-PAGE this was diluted 1 in 10 in 1xLB and 20 μl loaded per well. Uncleaved PA was diluted to the same extent and run separately.

2.5.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The Bio-Rad Mini Protean II Gel apparatus was used for SDS-PAGE. The apparatus was cleaned and assembled according to the manufacturer's instructions. The gels were made according to the method of Sambrook *et al.* (1989) at the required % acrylamide - 8% was used for most routine gels for PA and all gels had a 5% stacking gel. The higher concentration of ammonium sulphate used eliminated the need for degassing of the gel mix. A 10 well comb was inserted into the stacking gel. The gel was allowed to set, the comb removed and the wells washed out with water. The gel holders were assembled into the apparatus and inserted into the tank which was filled with running buffer (15.15 g Tris,

72.05 g Glycine, 5 g SDS, make to 5 litres). 5-25 μl of samples and appropriate markers (Dalton VII-L (Sigma) or pre-stained low range markers (Bio-Rad)) were loaded into the wells. The gel was electrophoresed at 200 V until the dye front had reached the bottom of the gels.

2.5.5 Protein staining

2.5.5.1 Coomassie staining

The proteins separated by SDS-PAGE could be visualised in the gel by simultaneously fixing and staining them with Coomassie Brilliant Blue in methanol:glacial acetic acid (0.25 g Coomassie Brilliant Blue R250, 45 ml methanol, 10 ml glacial acetic acid, 45 ml water). The gels were stained for at least 2 hours and the stain was replaced with destain solution (as above, less the dye) and left until the required amount of destaining had occurred. The gels were stored in sealed plastic bags in 10% glycerol.

2.5.5.2 Protogold staining

Western blots were stained to show protein bands by staining with a stabilised colloidal gold solution, Protogold (BioCell Research Laboratories, Cardiff). The manufacturer's instructions were followed. Silver enhancing was not found to be necessary.

2.5.6 Western Blotting

2.5.6.1 Electroblotting of proteins

During the PAGE, polyvinylidene diflouride (PVDF) membrane (Immobilon-P, Millipore, Watford) was wetted for 3 secs in methanol and then washed in water for 2 mins and placed into transfer buffer (25mM Tris, 192mM Glycine, 20% (v/v) Methanol pH 8.3). After electrophoresis the glass plates were split and the gels equilibrated in transfer buffer for 5 mins before being placed onto a filter paper pad. The gel was covered with the wetted PVDF membrane and another filter paper pad. This was placed between two fibre pads and sandwiched in a Bio-Rad Mini Trans-blot holder. The holder was replaced in the electrophoresis tank with an ice filled temperature stabilising compartment and electroblotted at 100 V for 1 hour. The gel was Coomassie stained to verify that the proteins had blotted across from the gel. The blotted proteins could also be visualised by running a duplicate gel onto PVDF membrane and then staining the protein bands with Protogold.

2.5.6.2 Detection of blotted proteins

The PVDF membrane was blocked in either 1% Blotto (1% (w v) Semi-skinned Milk Powder in PBS) or 5% Bovine Serum Albumin (BSA)-PBS for 30 mins at 37°C. All subsequent washes were performed with either 0.1% Blotto or 0.1% BSA-PBS respectively. The membrane was washed three times on a shaking table for 2 mins each. The membrane was incubated at room temperature for 1-3 hours on the shaking table in primary antibody diluted appropriately in 20 ml of washing solution. The membrane was washed 3 times for 5 mins each to remove the unbound primary antibody. The labelled secondary antibody was added for 1-3 hours, this was either a 1:100 dilution of gold labelled antibody in 5% gelatin, 0.1% BSA/PBS (Auroprobe BLplus) or a 1:1000 Horse Radish Peroxidase linked antibody (Sigma). If gold labelled antibody was used, the membrane was washed twice for 5 mins each and then washed twice for 1 min each in distilled water and equal volumes (7.5 ml) of enhancer and initiator solutions (IntenSE BL) added. For HRP-linked antibody the membrane was washed 5 times in PBS and then a fresh substrate solution of DAB (55 mg 3,3'-diaminobenzidine, a small crystal cobalt chloride, 100 μl of H_2O_2 in 100 ml PBS) added. The colour was allowed to develop and the reaction stopped by washing in excess water.

2.5.7 Colony Blotting

Colonies were picked and streaked onto duplicate plates. The streaks were about 1 cm long with approximately 20 streaks per plate. The plates were grown overnight. Circles of sterile nylon membrane (Hybond-N) were soaked in 100mM IPTG and allowed to dry. The membrane was placed over the plate and orientation marks drawn. The membrane was removed and placed colony side up on filter paper soaked in L-broth and antibiotic. The membrane was incubated for 1-2 hours at 37°C. The bacteria were lysed by suspending the membrane over chloroform in a bell jar for 30 mins. The membrane was then placed face down in a petri dish containing 10 ml of Tris-buffered saline (TBS - 8 g NaCl. 0.2 g KCl, 3 g Tris, add 800 ml water and adjust pH to 7.4 with HCl. make to 1 litre), 4 mg lysozyme and 100 μg DNAse I (Boehringer) and agitated on a shaking table. The membranes were washed twice in TBS and then blocked in 2% Blotto for 30 mins at 37°C. The membrane was washed in PBS and then incubated in the primary antibody - 1:1000 mouse anti-PA MAb in PBS - for 1 hour. The membrane was washed three times in PBS and then incubated with the secondary antibody - 1:1000 horse radish peroxidase labelled anti-mouse

IgG (Sigma)- for 1 hour. After washing 3 times in PBS the substrate DAB was added (see Section 2.5.6.2) and the colour developed. The membranes were washed and allowed to dry. Positive colonies were identified and linked to the duplicate plate streak.

2.5.8 Enzyme-linked Immunosorbent assays (ELISA)

2.5.8.1 LT-B

The ELISA method for LT-B and the anti-LT-B monoclonal antibody was provided by Dr. T Hirst (University of Kent). Microtiter plates were coated with 100 μl per well of 1.5 μg/ml of monosialoganglioside GM1 (Sigma) dissolved in PBS. Plates were incubated overnight and then stored at 4°C until required. Plates were washed 2x with PBS and blocked with 200 μl of 0.1% BSA dissolved in PBS at 37°C for 30 min. After 3xPBS washes the samples were added with serial dilutions in 0.1% BSA/PBS and a final well volume of 100 μl. The plates were incubated at room temperature for 60 min and then washed 3x in PBS/0.05% Tween-20. The anti-LT-B antibody was diluted 1:400 in 0.1% BSA/PBS and 100 μl added to each well and the plate incubated at room temperature for 60 mins. After 3x washes in PBS/0.055% Tween-20, 100 μl of a 1:1000 dilution of horse-radish peroxidase labelled goat anti-mouse IgG antibody (Bio-Rad) in 0.1%BSA/PBS were added and incubated for 60 min at room temperature. The plate was washed 3x in PBS/0.05% Tween-20. 30 mg of 2,2'-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (Sigma) were added to 50 ml of buffer (0.2M phosphate/citrate buffer pH 4.3) with 50 μl of H_2O_2. 100 μl of this substrate were added to each well and the plate read at 414nm in a Titertek Multiscan MCC/340 Mk II plate reader.

2.5.8.2 Anti-PA antibodies

Antibody titers against PA were measured using an ELISA by one of two methods. Mouse sera was tested by Dr PCB Turnbull, CAMR using an inhibition ELISA described in Turnbull *et al.* (1986). Briefly, a flat-bottomed 96 well microtiter plate was coated with PA (15 μg/ml PA in coating buffer - 0.16 g Na_2CO_3, 0.29 g $NaHCO_3$ made to 100 ml, pH 9.6) and allowed to dry. The wells were blocked with fetal calf serum, washed with PBS-0.0005% Tween 20 (PBST), and dried. For use, the plate was marked of in pairs of rows. 50 μl of PBST were added to the lefthand row of each pair of rows, and 50 μl of PA-PBST (15 μg/ml) to the righthand row of each pair of rows. In the first well of each lefthand row

add 25 μl of PBST, and add 25 μl PA-PBST to the first well of each righthand row. Add 25 μl of sample to both first wells and double dilute 50 μl down the row discarding the last 50 μl. Incubate for 30 mins at 37°C, wash and add a 1:800 dilution of HRP-labelled rabbit anti-mouse IgG (Sigma). After incubating at 37°C for 15 mins, wash and add 50 μl ABTS substrate (30 mg of ABTS added to 50 ml of 0.2M phosphate citrate buffer pH 4.3 with 50 μl of H_2O_2). Read Abs_{405} on a microtiter plate reader. The results are interpreted by looking for the last pair of wells which have a difference greater than 0.2 Abs_{405} units between the standard and inhibition rows.

The rabbit anti-sera was tested by Dr. ED Williamson, CBDE using a modification of the above technique. Inhibition rows were not run, the plates were blocked with 4% BSA in PBST and the secondary antibody was from Bio-Rad and was used at 1:1000. The endpoint was assumed to be 0.2 Abs_{405} units once the values had been zeroed against normal rabbit serum controls.

The second method was used to test for anti-PA antibodies from the in-house mouse experiment using the appropriate anti-mouse reagents and controls.

2.6 Animal Experiment methods

2.6.1 Preparation of anti-PA polyclonal antibodies

Two female New Zealand White rabbits weighing 3-5 kg were immunized with an initial injection of 0.25 ml of the human anthrax vaccine (DHSS, Russell Sq, London; Batch Number 330/E) given intramuscularly in two hindlimb sites. This was followed by two booster immunizations of 0.5 ml of vaccine at 2 and 4 weeks. As the anti-PA titres were only 1:640 at 6 weeks, two extra 0.5 ml immunizations were given at weeks 10 and 12 and the animals bled out at week 15. The blood was collected in 9.5 ml SST Gel and Clot activator Vacutainer tubes (Becton Dickinson, Maylan Cedex, France) and after the clot had formed, the tubes spun and the sera removed and stored at -20°C. The sera was tested for anti-PA antibodies by Dr. ED Williamson, CBDE.

2.6.2 Growth of cultures for inoculations

2.6.2.1 Preparation of seed cultures

The strains to be used for animal inoculations were grown overnight on agar plates containing antibiotics if required. A single colony was picked into 10 ml L-broth (with

antibiotic as required) and grown overnight. Sterile glycerol was added to 10% and the mixture aliquoted into sterile 1 ml screw-topped tubes which were frozen at -70°C as a seed culture. One tube from each batch was checked for *aro* and *his* phenotypes with the appropriate minimal media plates and for antibiotic resistance by plating out on L-agar and L-agar/ampicillin plates. Plates were checked for a smooth colony morphology and the absence of contaminants. The smooth phenotype of the *S. typhimurium* was also verified by showing the ability of the smooth phenotype sensitive phage P22 to adsorb to and lyse the bacteria and by analysing the LPS pattern on silver stained gels for differences between the wild type LPS and the seed cultures.

For P22 lysis, the bacteria were grown overnight and then mixed with appropriate dilutions of a P22 HT105/1 *int* transducing phage preparation. The infected bacteria were incubated for 45 mins at 37°C and then mixed with 3 ml of 0.7% top agar at 47°C and spread onto L-agar plates. The plates were examined for the appearance of plaques.

LPS was prepared from a whole cell suspension boiled in sample buffer and treated with 25 μg of proteinase K (Sigma) and then incubated at 60°C for 60 mins. The samples were analyzed by PAGE and stained with Bio-Rad silver stain kit according to the manufacturer's directions.

2.6.2.2 Preparation of inoculum for oral vaccination

For oral animal inoculations, one of the seed culture tubes was used to inoculate 100-400 ml of L-broth (with ampicillin if required). The seed culture tube was then discarded. The L-broth was grown at 37°C statically overnight. The Abs_{600} was measured and the cells pelleted and washed in ice-cold PBS. The cells were pelleted and then resuspended in an appropriate amount of PBS (assuming an Abs_{600} of 1 was equivalent to $2x10^9$ cells) to give twice the required cell density. This allowed for losses in the experimental procedure. The Abs_{600} of a 1:10 dilution was checked and the final volume of PBS revised to give the required cell density. An appropriate amount of cell suspension was aliquoted into an Universal flask and kept on ice until required. Duplicate serially dilutions in PBS of the cell suspension were made and aliquots plated out on duplicate plates and grown overnight. Colonies were counted and the actual viable count calculated. Plates were checked for a smooth colony morphology and the absence of contaminants.

2.6.2.3 Preparation of inoculum for IV vaccination

For IV animal inoculations, one of the seed culture tubes was used to inoculate 100 ml of L-broth (with ampicillin if required). The seed culture tube was then discarded. The L-broth was grown at 37°C statically overnight. The cells were pelleted and resuspended in 10 ml of sterile ice-cold PBS/10% glycerol. 0.7 ml aliquots were frozen at -70°C. To check the phenotype of the frozen inoculum, aliquots were defrosted at 37°C and plated out on L-agar and L-agar supplemented with ampicillin plates. Also the *aro* phenotype was checked by plating on minimal media plates with and without aromatic amino acid supplementation. The viable cell count of the frozen stock was determined by serial dilutions and spiral plating. Plates were checked for a smooth colony morphology and the absence of contaminants.

On the day of an experiment, a vial was defrosted at 37°C and diluted in ice-cold PBS to give $2.5x10^6$ viable cells/ml. A dose of 0.2 ml IV would therefore contain $5x10^5$ viable cells/ml. The actual count for the inoculum was verified by spiral plating a 1:100 dilution.

2.6.3 Method of inoculation

For oral (intra-gastric) inoculation, mice were lightly anaesthetized by placing them in a bell jar containing halothane soaked gauze beneath a metal screen. Once the mice had lost their righting reflex they were removed from the bell jar and held by the skin at the back of the neck so that the axis of the body was a straight line. A blunt ended needle on the end of a 1 ml syringe containing the inoculation dose, was gently inserted down the oesophagus to mid-thoracic level and the inoculation administered. The needle was withdrawn and the mouse allowed to recover from the anaesthetic.

For IV inoculation, mice were restrained with their tail exposed. 0.2 ml of the inoculum was injected into the tail vein.

2.6.4 Antibiotic selection *in vivo*

Ampicillin was used in some groups to attempt to maintain selection pressure so that plasmids would be maintained within the attenuated *S. typhimurium*. An ampicillin Trihydrate suspension (Penbritin Injectable suspension POM, Smithkline Beecham Animal

Health) containing 150mg/ml ampicillin was used. Mice were administered 50 μl (7.5 mg) SC once a day for seven days.

2.6.5 Vaccination with PA and rPA

Groups of mice were vaccinated with either PA (HPLC purified from *B. anthracis* culture supernatant) or rPA (purified from *E. coli* containing phagemid pORF1) mixed 50:50 with FIA. The PA/rPA was diluted in PBS to give a concentration of 0.2μg/μl. 50μl of this were mixed with 50μl of FIA and administered IP. Three doses were given 14 days apart.

2.6.6 Organ bacterial counts

At certain time points animals were euthanised by cervical dislocation and the animal's liver and spleen removed and placed in ice-cold PBS. The organs were placed separately into a Stomacher bag and 10 ml ice-cold PBS added. The organs were homogenised in a Stomacher Lab-Blender Model 80 (Seward Medical, London) for 2 min. Four 500μl aliquots, a 25 μl and a 100 μl aliquot of the homogenised organ were plated out on both L-agar plates and L-agar plates supplemented with ampicillin. The plates were incubated at 37°C overnight and colonies counted the next day. Depending on the colony density on the plates the total organ count was determined. The difference between the count on the L-agar and antibiotic supplemented plates was used to quantify the percentage of the bacteria which were still ampicillin resistant.

2.6.7 Serum anti-PA antibody levels

Mice were bled either from the tail vein or by cardiac puncture in anaesthetized mice. The blood was allowed to clot and the serum removed and stored at -20°C. The anti-PA antibody titre was determined by ELISA.

2.6.8 Challenge with virulent *B. anthracis*

All procedures with *B. anthracis* took place in approved Advisory Committee on Dangerous Pathogens (ACDP) Category III containment facilities within CBDE. The animals were maintained within a negative-pressure flexible film isolator for the 14 day challenge experiment.

B. anthracis Vollum 1B was grown from a frozen spore stock on nutrient agar plates. The bacteria were washed from the plates in sterile water and heated at 60°C for 60 minutes to kill vegetative cells. The spore preparation was washed by centrifugation. Serial dilutions were plated out to establish the spore count of the preparation which was found to be 10^9 spores/ml. The stock was kept in a glass container at 4°C. On 3 occasions the spore stock was diluted to give 500 spores/ml and this was plated out to establish the accuracy and reproducibility of the dilution method.

For challenge experiments, the spore stock was diluted on the day of the challenge and an appropriate volume injected SC in the mice. 1 ml of the diluted stock was plated out on nutrient agar plates and incubated overnight at 37°C. The colonies on the plates were counted to determine the actual cfu/ml count and hence derive the dose given.

Mice were checked twice a day. Any moribund mice were humanely euthanised. Deaths were confirmed as been due to anthrax by examination of blood smears from dead mice for evidence of the M'Fadyean reaction. Staining and examination of slides was carried out by Mr A Stagg (CBDE).

2.6.8.1 Diagnosis of anthrax - the M'Fadyean reaction

Blood films were made by clipping the tail of dead animals and smearing the blood onto a slide. The films were air-dried in the ACDP Category III isolator and then transferred to a Class III microbiological safety cabinet. The slides were heat fixed and stained for with polychrome methylene blue, which after 30 secs was washed off into hypochlorite solution. The slides were dried and examined microscopically for the reddish-purple capsular material and deep blue bacilli, an appearance referred to as the M'Fadyean reaction (Turnbull, 1990).

CHAPTER 3

Detection of *B. anthracis* by the Polymerase Chain Reaction (PCR) and PA by Western blotting

3.1 Introduction

This chapter describes the development of the Polymerase Chain Reaction (PCR) and methods for the analysis of PA by Western blotting which formed a background for the use of these techniques in this study.

PCR is a technique with many applications. Experience with this system was needed so that it could be used to detect the PA gene in recombinant organisms, assist in cloning precise fragments of the PA gene and also to identify the presence of recombinant organisms in animal tissue samples. Initial experimental work concentrated on using PCR to identify genes in the host organism *B. anthracis* and this experience was used to devise methods of identifying and amplifying the PA gene in other situations. The objective was to gain an insight into the requirements and limitations of the PCR.

There are a number of methods which could have been used for identifying the PA expressed from recombinant organisms. One possibility is an ELISA test which would measure the total amount of protein which was bound by the capture and detection antibodies used. This system is dependent on the specificity of the antibodies used and does not give any information on the size and fragmentation of the immunoreactive protein. Western blotting allows visualisation of immunoreactive protein bands which have been separated by PAGE. This method provides information on the fragmentation of the protein and so is the method of choice. The objective of this work was to evaluate the different antibodies available for Western blotting and assess the quantitative nature of Western blotting.

3.2 Detection of *B. anthracis* and recombinant clones by the PCR

Spores of *B. anthracis* are commonly found in the environment. They are also a potential Biological Warfare agent. One method of detection of these spores is using the PCR. This relies on the amplification of a gene unique to *B. anthracis* and preferably one which is required for virulence. The two important virulence factors are the toxin and the capsule which are encoded on plasmids pXO1 and pXO2. As the toxin is required for virulence, its encoding genes are possible targets for PCR. An initial study was made on the detection of spores by amplifying part of the edema factor (EF) gene from spore preparations. Experience with this was useful in designing PCR primers for PA and their

use to detect the PA gene in animal tissue samples and for the identification of recombinant clones containing an insert of the PA gene.

3.2.1 Design of primers homologous to parts of the EF and PA genes

PCR primers were designed to bind to the EF gene of *B. anthracis* and amplify a 1247 bp fragment of the gene. After extensive homology searches through GenBank, the primers used showed significant homology only with the EF gene (Carl *et al.*, 1992).

The PA gene is a preferred target for detection of virulent *B. anthracis* strains as it is an essential component of the anthrax toxin complex. Strains lacking the EF gene, but still producing Lethal toxin (PA and LF), are still virulent (Pezard *et al.*, 1991), but strains lacking a functional PA gene would have reduced virulence (Leppla, 1991; Pezard *et al.*, 1991). PCR primers ANTHPR3 and ANTHPR4 were originally designed for cloning purposes (see Chapter 5) and so were homologous to specific regions of the PA gene. These primers contained 5' tails which were not homologous to the PA gene but coded for restriction sites that would be incorporated into the PCR product. As these primers worked efficiently in PCR reactions with both plasmid and chromosomal DNA, their potential as PCR primers for detection was evaluated. For detection purposes primers ANPR3S and ANPR4S were made to contain only the homologous portion of the original primers. The sequence of the primers is shown:

	5' to 3' sequence	Length	CG ratio
ANPR3S	TCG AAC TCA AGA AAA AAG CGA	21	38%
ANPR4S	TAT CCT ATC TCA TAG CCT TTT TTA	24	29%

The CG ratio of the primers was constrained by the AT richness of *B. anthracis* DNA.

In order to assess the potential of these primers to hybridise with genetic material from other pathogenic organisms or environmental contaminants, a search of the GenBank/EMBL database (release 74, 102947 entries) was conducted by Mr K Martin (CBDE). The search was conducted to identify matches with the primers at an homology of 78% or greater. This search revealed 47 partial sequence matches but, as can be seen in Figure 3.1, the partial matches were well separated from the 100% homology of the primers with the PA gene suggesting that it would be possible to avoid false positives by adjusting

Figure 3.1. Homology between PCR primers and GenBank sequences

This figure illustrates the % homology between the PCR primers designed from the *B. anthracis* PA gene (ANPR3S and ANPR4S) and other nucleotide sequences contained within the GenBank/EMBL database (release 74, 102947 entries). The homology of the primers with the PA gene (100%), is shown at the bottom right of graph. The nearest homology is at 84% homology (ANPR4S and an unspecified GenBank sequence) which suggests that it would be possible to modify the annealing conditions of the PCR reaction to exclude false positives.

The homology searches were performed using the GENEMAN program, part of the DNASTAR software.

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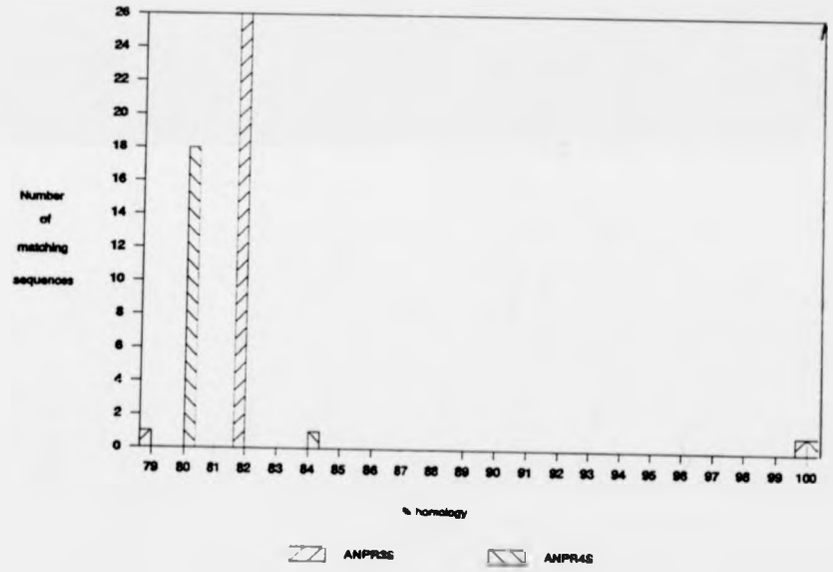

Figure 3.1 Homology between PCR primers and GenBank sequences

the stringency of the PCR reaction conditions.

3.2.2 Detection of *B. anthracis* spores using PCR primers homologous to the EF gene

A PCR using the EF primers generated a visible band on an agarose gel when used with a DNA template of 10^3 copies of a plasmid containing the gene or on the DNA released from 2 x 10^4 spores. If the product from a first round of the PCR was used as a template in a second PCR reaction, with primers that bind within the first PCR product (nested PCR), it was possible to produce a band from a dilution theoretically containing a single plasmid gene or 2 spores of *B. anthracis*. Various protocols were designed and tested to release the DNA from the spores so that it could act as a template in the PCR reactions.

3.2.3 Detection of PA gene in animal tissues

For the purposes of this study, the detection of the PA gene within animal tissues would allow screening of animal samples for recombinant *Salmonella* containing the PA gene. As the bacteria would be expected to persist within the liver and spleen, these tissues were chosen for this study. These organs contain many enzymes which, when the cells are lysed, may degrade DNA. Thus an initial study was made to see if the PCR would identify the PA gene in a spleen tissue sample. Normal mouse spleens were removed and homogenised in 5 ml of sterile water. Aliquots were inoculated with a colony of *S. typhimurium* either containing a plasmid with the PA gene or no plasmid. As the homogenate was found to coagulate on boiling, it was diluted. 5 μl of the spleen homogenates were mixed with 65 μl of water, boiled to lyse the bacteria, made to 100 μl with a PCR master mix, and subjected to 35 cycles of PCR with primers ANPR3S and ANPR4S. Negative controls included spleen homogenates without bacteria and water alone; the positive control was water inoculated with *Salmonella*. The controls gave negative and positive results as expected. Only two out of three positive inoculated spleen samples gave DNA bands of the expected size. This showed that the PCR reaction can take place in the presence of the diluted spleen material. As the spleens were initially homogenised in 5 ml of water, and tested at 1:1000 dilution, the concentration of positive bacteria within the spleen would need to be large enough to detect bacteria using this method. This would be expected to be the case in an animal clinically affected with anthrax, but with an oral dose of 1.6 x 10^{10} attenuated *S. typhimurium* SL 3261 the numbers of bacteria within the liver and

spleen drop to 10^2-10^3 bacteria per organ by 7 days post-infection (Maskell *et al.*, 1987). This would be close to the theoretical limit of detection of 1 bacteria in 5 μl of the spleen homogenate. It seems likely that to detect the PA gene in these circumstances a means of concentrating the bacteria (or the DNA) would be required.

3.2.4 Use of PA PCR primers in identifying recombinant clones

A simple method for identifying recombinant clones by PCR has been described by several authors including Sandhu *et al.* (1989). Following transformation of bacteria with ligated DNA, the bacteria were plated on selective media and grown overnight. Part of a colony which grew was picked from the plate, suspended in water or PCR reaction mix, boiled to lyse the cells and subjected to PCR with primers which would only produce an amplified band from DNA template containing the insert. If the primers are chosen so that one binds within the vector and the other on the insert, a product will only be seen if the insert is in the correct orientation.

This method was used to identify recombinant plasmids during several experiments as described in subsequent chapters. In one case false positive results were seen when colonies tested generated a PCR product but were subsequently proved not to contain the insert. These false positives were attributed to contamination of the agar plate with unligated insert from the original ligation mix. This could be transferred from the plate to the PCR reaction mix when the colony was picked from the surface of the plate. A protocol was established to treat bacteria suspensions with DNAse I prior to lysing the cells. This was shown to destroy insert DNA added to the sample, but not to prevent the PCR reaction producing a positive result with insert-containing recombinant clones. An alternative approach, which required a delay in analysing the samples, was to pick the colony and dilute it in 10 ml L-broth and grow overnight before removing 10 μl for analysis. This diluted out any contaminated DNA. False positive results were not seen if PCR primers were used which bound across the vector insert junction.

3.3 Detection of PA by Western blotting

3.3.1 Pre-stained Molecular Weight (MW) markers

In order to estimate the MW of immunoreactive bands after Western blotting, prestained MW markers (Bio-Rad, Low Range) were used in SDS-PAGE. Prestained

markers do not migrate within gels in the same position as the unstained proteins, and so are assigned apparent MWs by the manufacturer. During the course of this work it became evident that the apparent MW assigned to each marker did not agree with the expected size of the protein bands. To investigate this anomaly, and assign new apparent MWs to each band, dilutions of Sigma SDS 6H MW markers, a standard amount of prestained markers and trypsin cleaved and uncleaved PA were electrophoresed through an 8% SDS-PAGE gel. The amount of PA loaded approximated to that found in bands from whole cell lysates. The dilutions of Sigma MW markers were adjusted to give bands of similar intensity to the PA bands. To take into account any effects of blotting, the proteins were transferred using the standard protocol onto PVDF membrane. The Sigma MW markers and the PA were then visualised on the PVDF membrane by staining with Protogold protein stain. The developed blot is shown in Figure 3.2. The diluted MW markers (lane 3) gave bands of a similar colour density to the PA bands and so this lane was selected for a calculation of the apparent MWs of the prestained markers compared to known MW markers. A graph of log MW against relative mobility was plotted for the Sigma MW markers in lane 3 and used to estimate the apparent MW of the prestained markers in lane 6. The results gave new apparent MWs which are shown below (in kDa) and were used for this work. Other workers have reported similar apparent MWs for these markers and those quoted in Su *et al.* (1992) are shown.

Bio-Rad MW markers claimed size (kDa)	This study (kDa)	Su *et al.*, 1992 (kDa)
106	92	97.4
80	66	66.2
49.5	45	45
32.5	29	31

3.3.2 Polyclonal anti-PA Antibodies

Two types of anti-PA polyclonal sera were used in this study.

'Neptune' serum was raised in a horse given repeated injections of the Sterne live spore vaccine. This was produced at the Microbiological Research Establishment, Porton

Figure 3.2. Protogold stained blot to compare MW markers.

MW markers and PA were separated by SDS-PAGE (8% gel), transferred onto PVDF membrane and the membrane stained with Protogold.

Lanes 1 and 6 contained 10 μl and 20 μl respectively of Bio-Rad prestained MW markers, Low range. The four bands present were assigned apparent MWs (Da) by the manufacturer of: 106,000, 80,000, 49,500, and 32,500.

Lanes 2 to 5 contained 20, 10, 5 and 2 μl of Sigma 6H MW markers diluted 1:25 in water. The MWs (Da) are 205,000 (faint band 1.2 mm below blue line in lane 3), 116,000, 97,400, 66.000, 45,000 and 29,000.

Lane 7 contained 20 μl of uncleaved PA (approx 1.1 μg)

Lane 8 contained 20 μl of trypsin cleaved PA (approx 1.1 μg)

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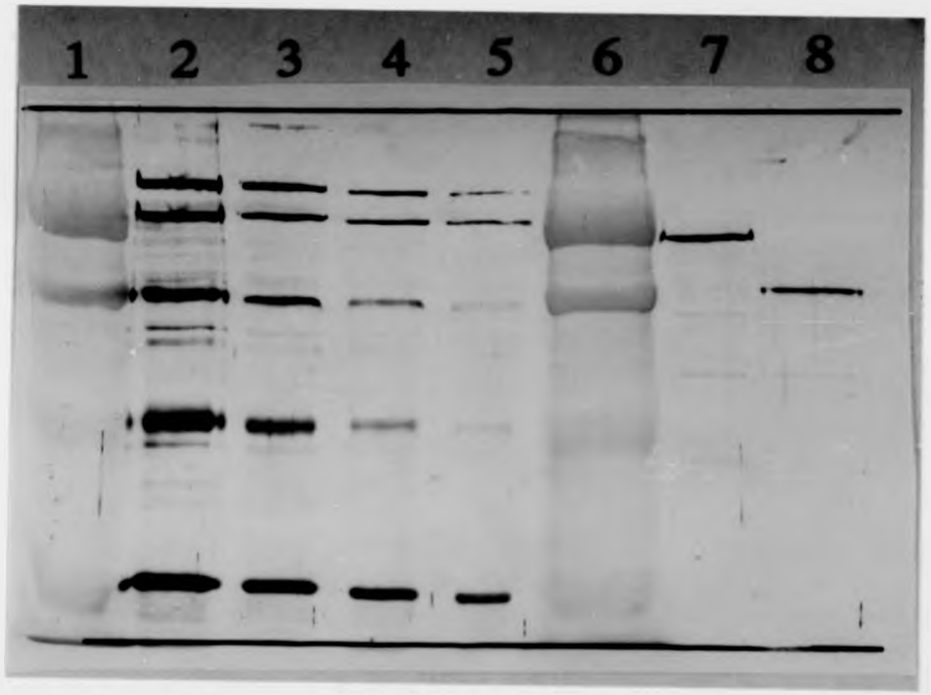

Figure 3.2 Protogold stained blot to compare MW markers

Down during the 1950's. This serum was not specific for PA but would be expected to contain antibodies to other *B. anthracis* antigens and antibodies against other antigens that the horse had been exposed to.

A more specific anti-PA serum was raised in rabbits during this study. The animals were given 5 injections of human anthrax vaccine which consists mainly of PA. After the second injection the anti-PA titres, measured by ELISA, were 1:640 and had risen to 1:3200 after the fourth injection, but dropped to 1:1600 after the fifth injection.

When these polyclonal sera were used as the primary antibody in Western blots, multiple bands appeared on the gels including bands in the control lanes. Also the level of background staining was high. This is shown in Figure 3.3. Quenching of the antibodies with sonicated extracts of *S. typhimurium* cells was tried but was not found to reduce background staining levels (results not shown). As the quantity of PA produced by recombinant clones in this study was easily visible on Western blots, the use of monoclonal antibodies was investigated to reduce the immunoreactive bands due to other antibodies present in the polyclonal sera.

3.3.3 Monoclonal anti-PA Antibodies

Three mouse monoclonal antibodies (3B6, 2D3 and 14C7) (Little *et al.*, 1988), obtained from the US Army Medical Research Institute for Infectious Diseases, were evaluated for their suitability as primary antibodies for Western blots. The hybridomas secreting the antibodies were produced using different immunization schedules with purified PA. *In vivo* neutralization studies showed a significant delay of time to death (2D3) or survival (3B6 and 14C7) in rats after IV injection of the MAb and lethal toxin (Little *et al.*, 1988). 3B6 and 14C7 were assumed to recognise the same antigenic region on the basis of competitive-binding experiments and shown to inhibit binding of PA to cells. These two MAbs are thought to bind to the cell-receptor site which has been localized to the C-terminal end of PA (Singh *et al.*, 1991). Deletions at the C-terminal end were shown to produce PA that was not recognised by these two MAbs (Little & Lowe, 1991). The binding site for 2D3 differs from 3B6/14C7 and has not been identified. It has been reported that these MAbs do not bind to PA after immunoblotting from SDS-PAGE gels (Singh *et al.*, 1991) but this effect was not seen in this study. It is possible that this was due to the PA re-configuring during the Western blotting process in the absence of SDS in the blotting buffer.

Figure 3.3. Western blots with polyclonal anti-PA antibodies

Whole cell lysates were made of *S. typhimurium* SL 3261 with pBluescript (lane 1) or pVET4M (lane 2). Trypsin-cleaved PA was diluted 1:10 with 1xLB (lane 3). 10 μl of each were loaded and two repeats of this were made. 10 μl of Bio-Rad prestained MW markers, low range, were loaded on the lefthand side of the gel and the proteins separated by SDS-PAGE (8% gel). Apparent MWs are marked beside the gel. The proteins were transferred onto PVDF membrane, the repeats cut into two strips and treated with either a 1:500 dilution of Neptune (Horse) sera or 1:1000 dilution of rabbit anti-PA sera. The appropriate HRP conjugated anti-species IgG was used as the secondary antibody at 1:1000. The substrate DAB was used to detect the secondary antibody.

The Neptune sera was from a horse vaccinated with the live spore vaccine. The rabbit sera was from a rabbit vaccinated with the human anthrax vaccine which consists mainly of adjuvanted PA.

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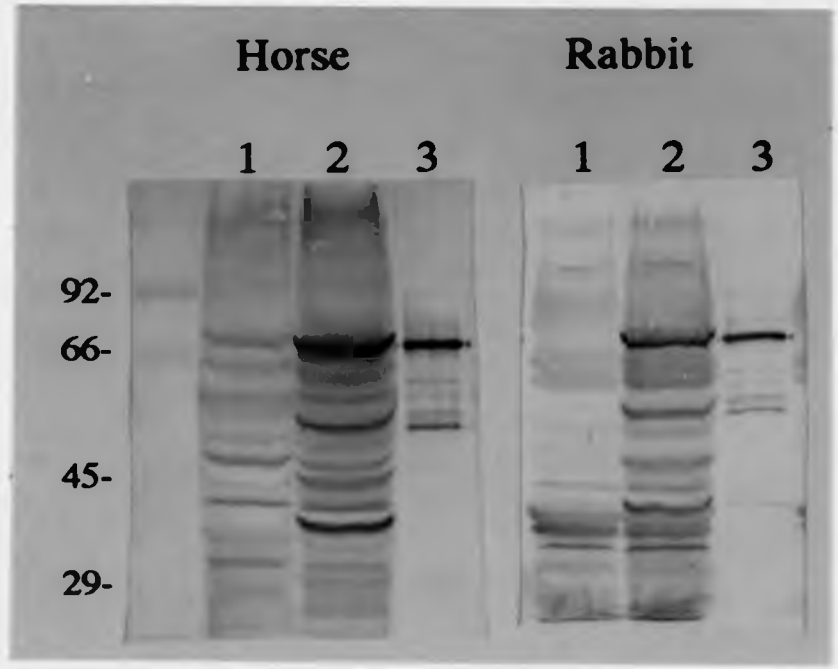

Figure 3.3 Western blots with polyclonal anti-PA antibodies

As two of the MAbs bind to a site at the C-terminal end it would be expected that they would only bind to PA-83 or fragments which contain the C-terminal end.

The MAbs were used in Western blots separately as shown in Figure 3.4. It was found that the MAbs bound to both cleaved *B. anthracis* PA and a recombinant PA-63 which has a modified C-terminal (addition of 5 amino acids, see Chapter 5). The MAbs which bind to the C-terminus of PA show only the PA-63 band in the cleaved PA and the PA-20 band is not visualised. The recombinant PA-63 is degraded and so shows multiple bands. As the 3 MAb produced similar patterns, in most routine blots the three MAbs were used together at a 1:1000 dilution.

3.3.4 Quantitisation of band intensity in Western blots

As it was necessary to make quantitative comparisons between immunoreactive bands of PA on Western blots the relationship between band intensity and the amount of PA present was investigated. This experimental work also allowed an estimate of the limit of detection of PA by Western blotting using the mouse MAbs.

Doubling dilutions of cleaved PA were electrophoresed through an 8% SDS-PAGE gel, transferred onto PVDF membrane and reacted with a primary antibody (mouse anti-PA monoclonals) and a secondary antibody of rabbit anti-mouse IgG conjugated to HRP and developed with DAB. The result of this experiment is shown in Figure 3.5. The dilutions of PA chosen for the experiment produced bands of similar intensity to that seen in whole cell lysates of recombinant clones. On the original blot a faint band was also visible in lane 7, and the limit of detection was concluded to be between 26 and 52 ng of PA. To compare band intensity and quantity of PA more precisely, the PVDF membrane was scanned in a densitometer and the peak areas for the bands plotted against lane numbers (Figure 3.6). The relationship between the peak area and amount of PA was linear over the range 103 to 825 ng of PA. However, due to possible differences in staining intensity between blots, only bands on the same blot were compared using this method.

3.4 Conclusions

The objectives of this chapter were to develop the techniques of the PCR and Western blotting for use in this study. PCR analysis was required to detect the presence of specific genetic material and Western blotting to detect the protein product.

Figure 3.4. Western blots with monoclonal anti-PA antibodies

Whole cell lysates were made of *S. typhimurium* SL 3261 with pBluescript (lane 1) or pVET4M (lane 2). Trypsin-cleaved PA (PA-63 and PA-20) was diluted 1:10 with 1xLB (lane 3). 10 μl samples were loaded on a gel and three identical repeats of these three lanes were separated by SDS-PAGE (8% gel). 10 μl of Bio-Rad prestained MW markers, low range, were loaded on the lefthand side of the gel. Apparent MWs are marked beside this lane. The separated proteins were transferred onto PVDF membrane which was cut into strips and reacted with a 1:1000 dilution of each monoclonal antibody - 14C7, 2D3 and 3B6 (Little *et al.*, 1988). Anti-mouse HRP conjugated was used as the secondary antibody at 1:1000. The substrate DAB was used to detect the bound secondary antibody.

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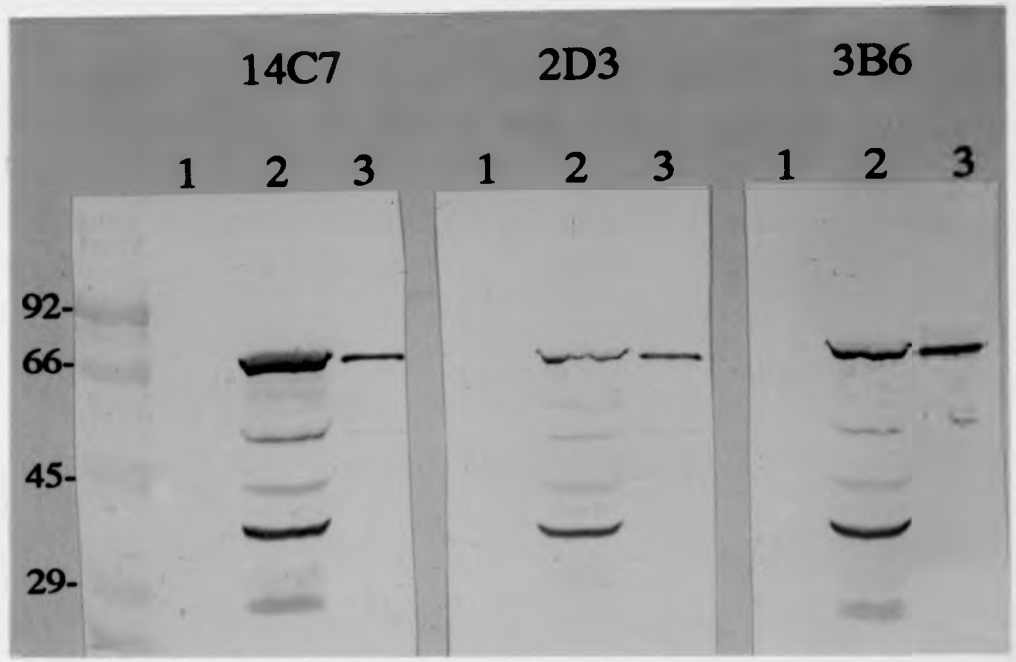

Figure 3.4 Western blots with monoclonal anti-PA antibodies

Figure 3.5. Doubling dilutions of PA

PA was cleaved with trypsin and diluted to give a concentration of 110 ng/μl. 15 μl was loaded onto lane 1 and doubling dilutions made and loaded onto lanes 2 to 9. The amount of PA loaded in each lane was:

Lane	1	2	3	4	5	6	7	8	9
ng	1650	825	412	206	103	52	26	13	7

The PA was separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane, incubated in mouse anti-PA monoclonal antibodies, secondary antibodies of rabbit anti-Mouse IgG conjugated to HRP and developed with DAB.

Bio-Rad prestained MW markers were separated on the left of the gel (lane MW). The position of the markers is shown by four dashes on the left of the photograph and the apparent MWs of the markers, from the top, is 92 kDa, 66 kDa, 45 kDa and 29 kDa.

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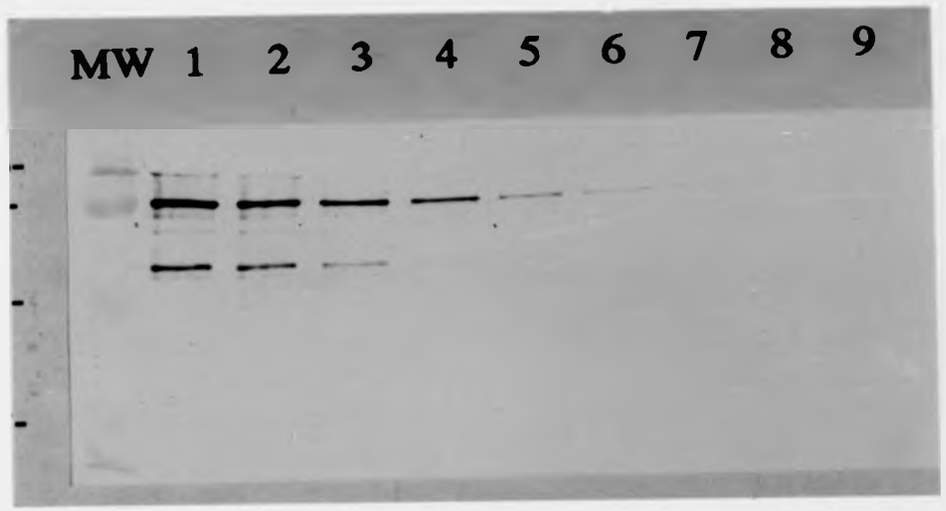

Figure 3.5 Doubling dilutions of PA

Figure 3.6. Peak areas of doubling dilution bands of PA

The PVDF membrane shown in Figure 3.5 was scanned on a LKB 2222-010 Ultroscan XL Laser Densitometer (LKB, Bromma, Sweden) and analyzed using LKB 2400 GelScan software. The bands were scanned at 1 and 5 times 800 μm scan widths and the peak areas (AU/mm) for the bands in lanes 1 to 5 plotted. The peak area was determined using the signal (or dropline) integration method with each peak assigned its own baseline with the valley option. The peak area was plotted against the lane number on Figure 3.5.

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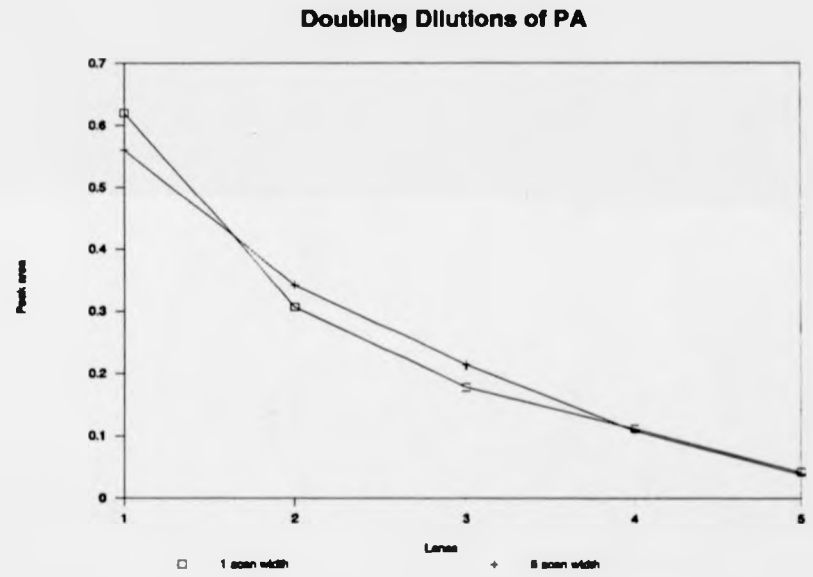

Figure 3.6 Peak areas of doubling dilution bands of PA

It has been shown that the PCR has the potential to detect the presence of *B. anthracis* genetic material in environmental samples. Although initial studies used primers for the EF gene, it may be more logical to use primers against the PA gene in order to detect *B. anthracis* spores as PA is required for both edema toxin and lethal toxin function. The *B. anthracis* PA gene is thought to be unique and a related toxin has not been identified in other bacteria. The PA gene is located on the plasmid pXO1 and it's sequence is believed to be conserved between all virulent strains of *B. anthracis*. Attenuated strains may lose this plasmid, but all virulent strains contain this plasmid. As the PA gene is located on pXO1, detection of this gene should be limited to bacteria which have retained the plasmid and so are potentially virulent. The capsule is also required for full virulence and the *cap* genes are located on the plasmid pXO2. Makino *et al.* (1993) have recently published details of a PCR detection method using primers which bind to the *capA* gene. PCR with primers designed to bind to the PA gene and to one of the *cap* genes could be used to detect the presence of both plasmids and so confirm the fully virulent plasmid genotype.

Candidate primers for detection of the PA gene have been identified and tested. They have been screened for cross-reactions against sequenced genes with a computer search. The search procedure identifies homology without differentiating which region of the primer is homologous. Even with a low percentage match between a primer and template, if the homology occurs at the 3' end of the primer, the primer could still produce a product in a PCR. If the match is higher, but the 3' end is not homologous, then the primer would not function well in the PCR. Thus the overall homology of the primers may not give an accurate assessment of their ability to act as PCR primers. Also a primer can only function in a PCR if there is a second primer bound to the opposite strand within a suitable distance of the first. The requirement for two primers to bind appropriately within the same region reduces that possibility of false positives. Before use in a test situation, the candidate primers could be screened against a DNA test panel from related bacteria and ubiquitous environmental bacteria. Uncontaminated *B. anthracis* samples can be identified relatively easily by culture and staining, but this method is less reliable when contaminated samples are analyzed using selective media. PCR can detect the presence of *B. anthracis* genetic material in contaminated samples and also can detect it in non-viable samples, if the DNA is intact.

The PCR can detect bacteria (*B. anthracis* and recombinant bacteria) which contain the PA gene in animal tissue samples. In samples from clinical cases of anthrax, large numbers of *B. anthracis* organisms will be present. These could be detected by culturing (if still viable), or by using the PCR. As the PCR can detect genetic material from non-viable organisms, this techniques could have a role in diagnosing disease in sterile specimens. In an unopened carcase, viable *B. anthracis* may be destroyed by putrification and PCR analysis may allow a diagnosis to be made. In the case of recombinant *S. typhimurium*, the number of bacteria which may be present in a tissue sample could be below 100. As the sample must be diluted to lower the protein content in the PCR reaction, the threshold for detection by the PCR may be exceeded. In this case culturing methods may be more sensitive.

The bacteria in the spleen samples were lysed by boiling to release the template DNA. Makino *et al.* (1993) showed that a SDS, Sarkosyl and proteinase K lysis method gave a more sensitive result. Their method involved a 3 hour clearing incubation and three phenol-chloroform extractions and an ethanol precipitation. They were able to easily identify 10^3 spores in a spleen sample, although their lysis and PCR method took eight hours in total. Their boiling method was quicker (4 hours) but could only detect 10^6 spores. It is therefore possible to increase the sensitivity of the technique, but at the expense of time and simplicity.

PA PCR primers are of use in identifying recombinant clones following ligations. This method allows the rapid screening of many clones and is particularly useful in blunt-end ligations where primers can be designed so that the product will extend across the vector-insert junction and so only identify inserts orientated in the desired direction. False positives can occur if the PCR primers only bind to the insert and the PCR reaction mixture becomes contaminated with unligated insert DNA, but various strategies can be used to overcome this.

Western blotting was chosen for the detection of expressed PA because it could reveal information on both the quantity and quality of the protein. There is no simple test for the biological activity of PA that could have been used for quantisation. ELISAs can be performed quickly and can be 10 to 100 fold more sensitive than Western blotting (Harlow & Lane, 1988), but in this study speed and sensitivity were not an issue. An ELISA would only provide information on the total amount of bound antigen and, depending on the

capture and detection antibodies used, the same reading could be obtained with full-length PA and degraded PA. Western blotting would show this difference, but the limitations imposed by the antibodies used must be understood. A polyclonal sera might contain antibodies which bind to many sites on the protein. Thus this sera would detect most fragments when transferred onto a membrane. Monoclonal antibodies only bind to one epitope and so will only identify the fragments which contain that site. Thus with monoclonal antibody in a Western blot the full range of fragments will not be detected. If different monoclonals are used, which bind to different sites on the protein, a wider range of degraded protein bands will be detected. Polyclonal sera may contain antibodies which bind to other proteins in a cell lysate and so give non-specific bands and also a higher background staining level. Monoclonal antibodies have the advantage of only detecting the protein of interest and of usually giving lower background staining. This allows a more accurate assessment of band densities.

When using Western blotting prestained MW markers can be used to estimate the MWs of immunoreactive bands. The MW proteins are conjugated to a dye and this alters the electrophoretic properties of the molecule. The proteins migrate through the gel at a different rate to the unconjugated protein and, for use in Western blotting, must be assigned apparent MWs by calibrating the markers against known MW markers. The manufacturer's apparent MWs of the prestained markers used were found to be inaccurate in this laboratory. This may be related to the type of gel used or the conditions used during the PAGE. Therefore new apparent MWs have been determined and are used in this study. Horse polyclonal anti-*B. anthracis* antiserum was available at CBDE but a more specific anti-PA antiserum was raised in rabbits. Both polyclonal sera were shown to produce non-specific bands in Western blotting and high levels of background staining. The use of three mouse monoclonal anti-PA antibodies was investigated and found to give a clearer blot. Two of the MAbs (3B6 and 14C7) were known to bind to PA-83 and PA fragments which contain the antibody binding site near the C-terminus. Thus N-terminus fragments would not be detected. As the other MAb (2D3) produces a similar banding pattern it probably also binds to a site close to the C-terminal end of PA, otherwise other fragment bands would be visible. When the three MAbs were used in combination, a similar banding pattern was seen. As only limited amounts of the MAbs were available, they were used together to provide sufficient quantity of a known mixture for this study.

Doubling dilutions of PA were Western blotted. Densitometer scanning of transferred dilutions of PA produced a graph of peak areas against amount of PA and showed the relationship between these parameters. This shows the feasibility for a comparison of band intensity on a Western blot.

The information determined in these experiments was used as a basis for experimental procedures described in subsequent Chapters.

CHAPTER 4

Effects of copy number and promoter on expression of 83 kDa PA

4.1 Introduction

To produce a potential vaccine strain PA must be stably expressed in the attenuated *Salmonella* strain. The coding sequence for PA was available on a pBR322 based plasmid designated pPA26 (see section 4.2.1) which expressed PA at low levels in *E. coli*. The initial strategy to increase expression was to replace the *B. anthracis* PA promoter with an *E. coli* promoter which would be expected to function at a higher level in both *E. coli* and *S. typhimurium*. All the constructs made are shown on a fold-out page at Annex A. It was decided to clone the ribosome binding site (RBS) and open reading frame (ORF) of PA (from pPA26) after the *lac* promoter in the phagemid pBluescript II. As the replicon of pBluescript has a higher copy number compared to pBR322, this construct would probably also increase the copy number of the PA gene. Thus it was necessary to investigate the consequences of increasing the gene copy number on expression of PA. Therefore the first construct made contained the pPA26 insert in pBluescript (designated pCB1). Expression from this clone was compared to pPA26 and related to plasmid copy number. Secondly a construct with the RBS and ORF of PA after the *lac* promoter was constructed (pORF1) to compare the expression from this promoter and the *B. anthracis* PA promoter. The constructs were initially placed into *E. coli*, but subsequently moved into the *aroA* *S. typhimurium* strain SL 3261 which can be used as a vector in mice.

4.2 Copy number effects

4.2.1 Plasmid pPA26

Plasmid pPA26 was derived from plasmid pSE36 (Vodkin & Leppla, 1983) which consists of pBR322 with a 6 kbp *Bam*HI fragment from the *B. anthracis* plasmid pXO1. Plasmid pPA26 was constructed from pSE36 by a partial *Hin*dIII digestion to leave a 4.235 kbp insert which contained the PA gene. Plasmid pPA26 (Welkos *et al.*, 1988) had a DNA insert which contained the *B. anthracis* promoter, signal sequence and ORF for PA. The insert DNA also contained an ORF for an uncharacterised protein upstream from the PA ORF (Welkos *et al.*, 1988). Plasmid pPA26 is illustrated in Figure 4.1 and a schematic representation of the DNA and the relevant restriction sites is shown in Figure 4.2.

Figure 4.1 Plasmid pPA26

A. Plasmid pPA26 (Welkos *et al.*, 1988) contains a *Hin*dIII-*Bam*HI insert of *B. anthracis* DNA in the vector pBR322 to give a total size of 8.25 kbp.

B. The *B. anthracis* DNA insert is shown. Within the insert is the open reading frame (ORF) for the PA gene located towards the 3' end of the insert. The ORF codes for the full-length (83 kDa) PA which can be cleaved by Trypsin into a N-terminal 20 kDa fragment and a 63 kDa C-terminal fragment.

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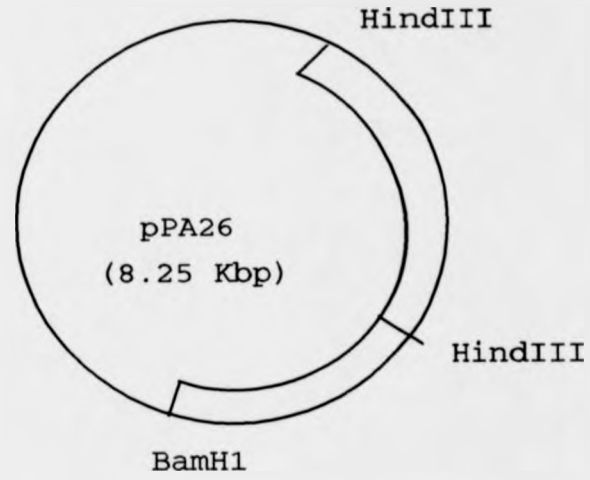
A.



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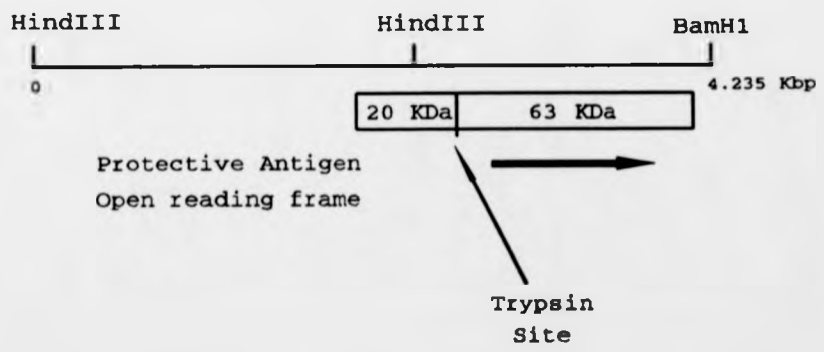

Figure 4.1 Plasmid pPA26

Figure 4.2 pPA26

A. This represents the regulatory and coding regions of the PA gene in plasmid pPA26. This pBR322 based plasmid contains the *B. anthracis* promoter followed by coding regions for the signal sequence and full-length PA which can be cleaved by trypsin into 20 and 63 kDa fragments.

B. This shows the relevant restriction enzyme sites in plasmid pPA26. The *Hin*dIII-*Bam*HI *B. anthracis* DNA insert in pBR322 is shown as a box. It contains an internal *Hin*dIII site and the ORF for PA. There is a *Cla*I site in pBR322 upstream from the insert.

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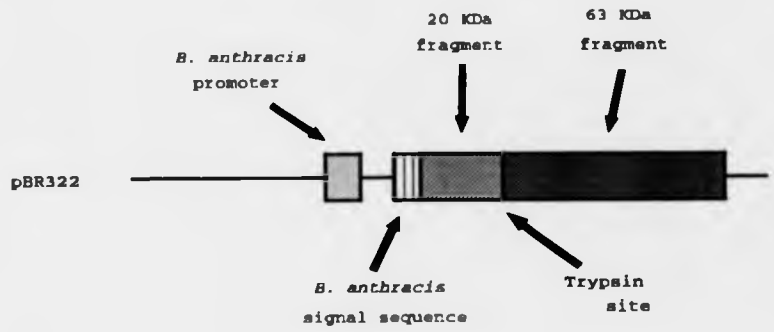
A.



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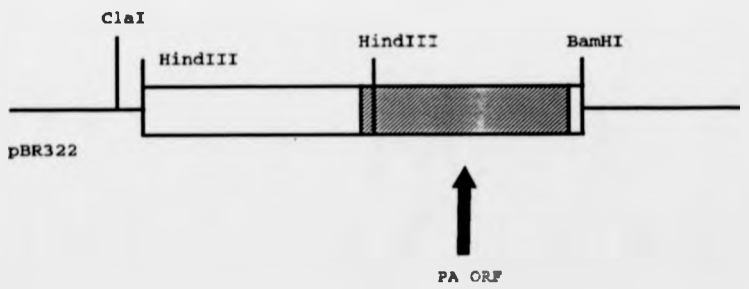


Figure 4.2 pPA26

4.2.2 Construction of phagemid pCB1

In order to evaluate the effects of copy number differences between pPA26 and other constructs, the insert from pPA26 was moved into the vector pBluescript II KS+. The phagemid pBluescript allows the simple isolation of ssDNA for sequencing. It also has many flanking restriction sites if the insert needs to be moved subsequently. pBluescript can be grown in *E. coli* which carry the *lacI*^{q} gene on the F' episome thus repressing the *lac* promoter. Expression can be induced with IPTG as required. The constructs can be moved to *S. typhimurium* SL 3261 where the *lacI*^{q} gene is absent and expression will be constitutive. This is necessary for a candidate vaccine strain and would not be possible if *lacI*^{q} was present on the plasmid.

The pPA26 insert contains external *Hin*dIII and *Bam*HI sites but also an internal *Hin*dIII site. In order to avoid the difficulties of a partial *Hin*dIII - *Bam*HI restriction digest, a *Cla*I - *Bam*HI fragment was cut from pPA26 and force cloned in *Cla*I-*Bam*HI cut pBluescript II KS+. This construct was also made by Little & Lowe (1991) as a starting point for their investigation of C-terminal deletions. This is illustrated in Figure 4.3. Following the restriction endonuclease digestions of pPA26 with *Cla*I, *Bam*HI and *Pvu*II (to cut the vector DNA into smaller fragments) and of pBluescript with *Cla*I and *Bam*HI, the appropriate bands were cut from agarose gels and purified using Qiaex. The vector and insert were ligated overnight, electroporated into *E. coli* and colonies which grew on L-agar plates supplemented with ampicillin were picked. These were grown up in 10 ml L-broth supplemented with ampicillin and mini-prepped. A clone with the appropriate sized insert was designated pCB1 and a large scale Qiagen plasmid preparation was made. The plasmid was restricted with several enzymes and cut as expected.

4.2.3 Comparison of pPA26 and pCB1

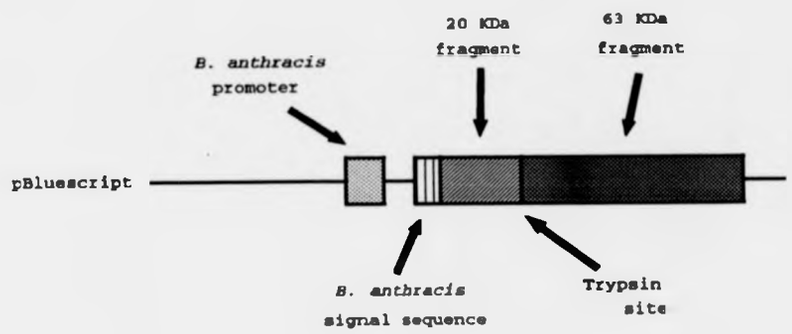
Expression of PA from pPA26 was low and difficult to detect by Western blotting. In order to easily detect the expressed protein it is necessary to use a method designed to enhance expression. The method uses chloramphenicol to amplify the number of recombinant plasmids per cell before washing out the antibiotic and allowing protein synthesis to recommence (Little & Lowe, 1991). The maximal plasmid gene copy number increases the ratio of plasmid proteins to cell proteins and makes detection easier. The plasmid copy number reached will depend on the plasmid replicon. This method was used

Figure 4.3 pCB1

A. Phagemid pCB1 was constructed to contain the *B. anthracis* DNA insert from the pBR322 based plasmid pPA26 in the phagemid pBluescript II KS+. This diagram shows that the PA ORF is still downstream from the *B. anthracis* PA promoter region and not the *lac* promoter of pBluescript.

B. This shows the relevant restriction enzyme sites in phagemid pCB1. The box represents the *Cla*I-*Bam*H1 insert from pPA26 that was cloned into pBluescript. The PA ORF is shown with internal *Hin*dIII and *Pst*I sites.

A.



B.

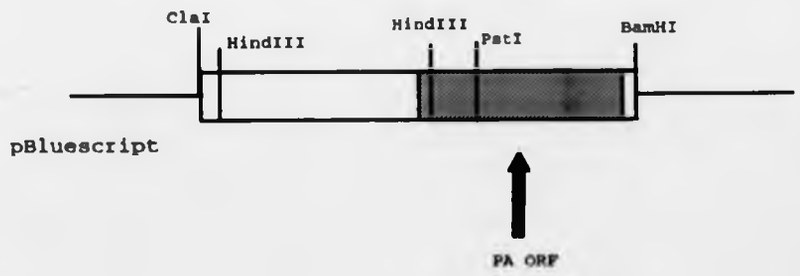


Figure 4.3 pCB1

so that the PA produced could be visualised on a Western blot and compared to plasmid copy number. As this method selects for maximal copy number, it does not necessarily represent the plasmid copy number ratio that might exist in normally growing cells.

4.2.3.1 Plasmid copy number

10 ml cultures of *E. coli* JM 109 containing either pBluescript, pPA26 or pCB1 were grown overnight and used to seed flasks of L-broth supplemented with ampicillin. Following initial growth, chloramphenicol was added. The next day the cells were washed and grown for 1 hour. From the Abs_{600} readings of the cultures, the densities were adjusted with L-broth to give equal Abs_{600} readings. Duplicate Qiagen column plasmid preparations were made of the *E. coli* JM 109/pPA26 and pCB1 cultures using columns with a 20 μg capacity. For pPA26, 3 ml of culture were used for each preparation and the plasmid pellets were resuspended in 20 μl of water. For the pCB1 cultures only 1.5 ml of culture was used (as the copy number was expected to be higher) and the pellets were resuspended in 50 μl of water. 20 μl of each plasmid solution were run on a agarose gel as shown in Figure 4.4. From the known amount of MW markers loaded, it was estimated that the bands present related to a total plasmid preparation content of about 1.25 μg of plasmid which is well within the capacity of the 20 μg Qiagen column used, and so column overloading should not have been a problem. Owing to the different culture volumes and dilutions used, the amount loaded in the pPA26 lanes equates to 5 times more than in the pCB1 lanes. The gel was photographed and the negative scanned on a densitometer to calculate areas for the bands of:

Plasmids	pPA26		pCB1	
Areas of bands	0.134	0.132	0.239	0.282
Average areas	0.133		0.2605	
Corrected for amount loaded	0.133		1.3025	

This experiment showed that on a cell for cell basis there was 10 times more of the pCB1 plasmid than of pPA26. This order of magnitude difference is not unexpected as the copy number for pBR322's pMB1 replicon is approximately 15-20 and for the pUC based

Figure 4.4 Duplicate plasmid preparations of pPA26 and pCB1

Duplicate plasmid preparations of *E. coli* JM 109 chloramphenicol amplified cultures were made using Qiagen columns. 20 μl aliquots were run on an 0.8% agarose gel with 4 μl of BCL DNA MW markers III. The gel was photographed. From the left of the gel are the duplicate pPA26 plasmids, then the duplicate pCB1 plasmids with the MW markers on the right. Due to differences in the amount of medium used and the volume of water used to resuspend the DNA pellet (explained in the text) the plasmid present in the pPA26 lanes represents that recovered from five times more cells than that present in the pCB1 lanes.

In order to verify that the Qiagen columns were not over loaded, a calculation of the amount of DNA recovered was made. The top band present in the MW marker lane represents 428 ng of DNA (based on the manufacturers stated marker concentration). From this it is estimated that the pCB1 band contains approximately 500 ng and so the total preparation would have contained 1.25 μg. This is well within the loading capacity of the 20 μg Qiagen column used.

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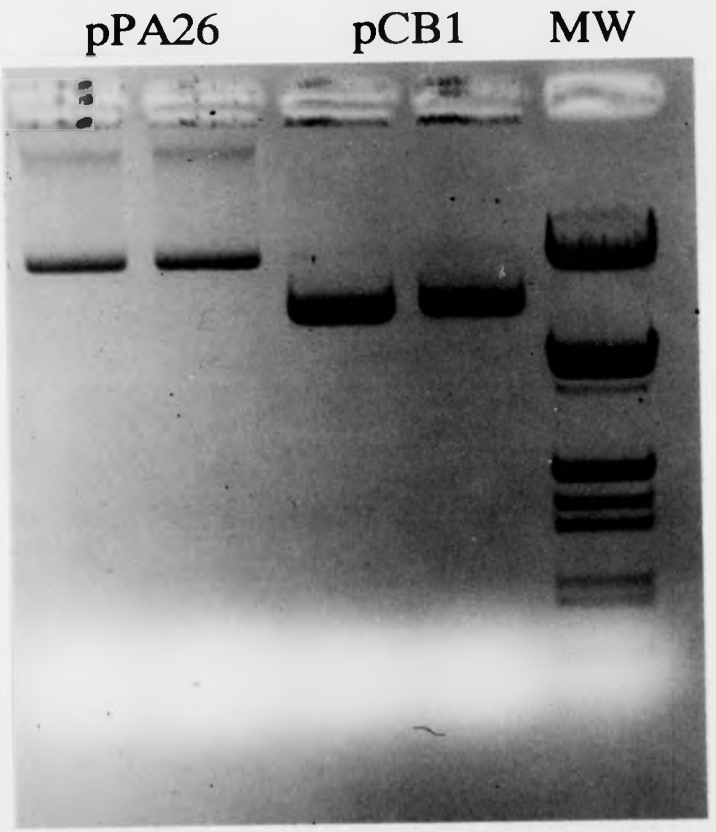

Figure 4.4 Duplicate plasmid preparations of pPA26 and pCB1

pBluescript II pMB1 replicon it is 500-700 (Sambrook *et al.*, 1989). The difference in copy number is attributed by Minton *et al.* (1988) to a G to A mutation which alters the transcription start of RNA I and so affects its binding to RNA II thus relaxing copy number control.

4.2.3.2 PA expression

The same cultures used for plasmid copy number preparations were used to provide whole cell lysates for Western blotting. 15 ml of the cultures were pelleted and resuspended in 1 ml of loading buffer. The lysate was boiled and a fraction corresponding to 3 x 10^6 cells subjected to SDS-PAGE and Western blotting with mouse MAbs. A photograph of this gel is at Figure 4.5. The double band in lanes 2 and 3 was suggested by Little & Lowe (1991) to represent PA with and without the signal sequence. However the signal sequence is only 29 amino acids in length (Welkos *et al.*, 1988) and would only correspond to a 3 kDa difference. Conformational changes between the two species may account for a further spread between the bands. This is discussed in Chapter 6. Faint breakdown product bands are also seen on the gels. The bands present were scanned on a densitometer but the double band and staining between the bands did not give a clear graph and the peaks could not be quantified this way. However it is clear that the pCB1 lane contained more PA than the pPA26 and this increase could be of the same order of magnitude as the 10 times increase in plasmid copy number.

4.3 Promoter effects

To investigate the effect of changing the promoter from the *B. anthracis* PA promoter to the *E. coli lac* promoter, the ribosome binding site, signal sequence and ORF for PA was cloned into pBluescript after the *lac* promoter and a short region of the β-galactosidase sequence. The phagemid was designated pORF1.

4.3.1 Construction of phagemid pORF1

The construction of pORF1 is illustrated in Figure 4.6. Two PCR primers were designed which bound to either end of the required region in pPA26. Each primer also had a non-complimentary 5' tail which contained appropriate restriction sites for cloning the PCR product into pBluescript. This is illustrated in Figure 4.7. The upstream primer (PAALL5)

Figure 4.5 Western blot comparing PA expression from pPA26 and pCB1

Whole cell lysates from chloramphenicol amplified cultures of *E. coli* JM 109 carrying either pBluescript (as a negative control), pPA26 or pCB1 were loaded on a 8% SDS-polyacrylamide gel. The amount loaded was between 6 and 10 μl related to the cell density of the original cultures thus each lane represents approximately 3 x 10^6 cells. 10 μl of Bio-Rad prestained MW markers were loaded on the lefthand side of the gel and apparent MWs (kDa) are noted beside them. The gel was blotted onto PVDF membrane, incubated in a primary antibody solution of mouse monoclonal anti-PA antibodies and a secondary antibody solution of HRP-linked anti-mouse antibodies. The bands were visualised with DAB.

Lane 1 *E. coli* JM 109 / pBluescript
Lane 2 *E. coli* JM 109 / pPA26
Lane 3 *E. coli* JM 109 / pCB1

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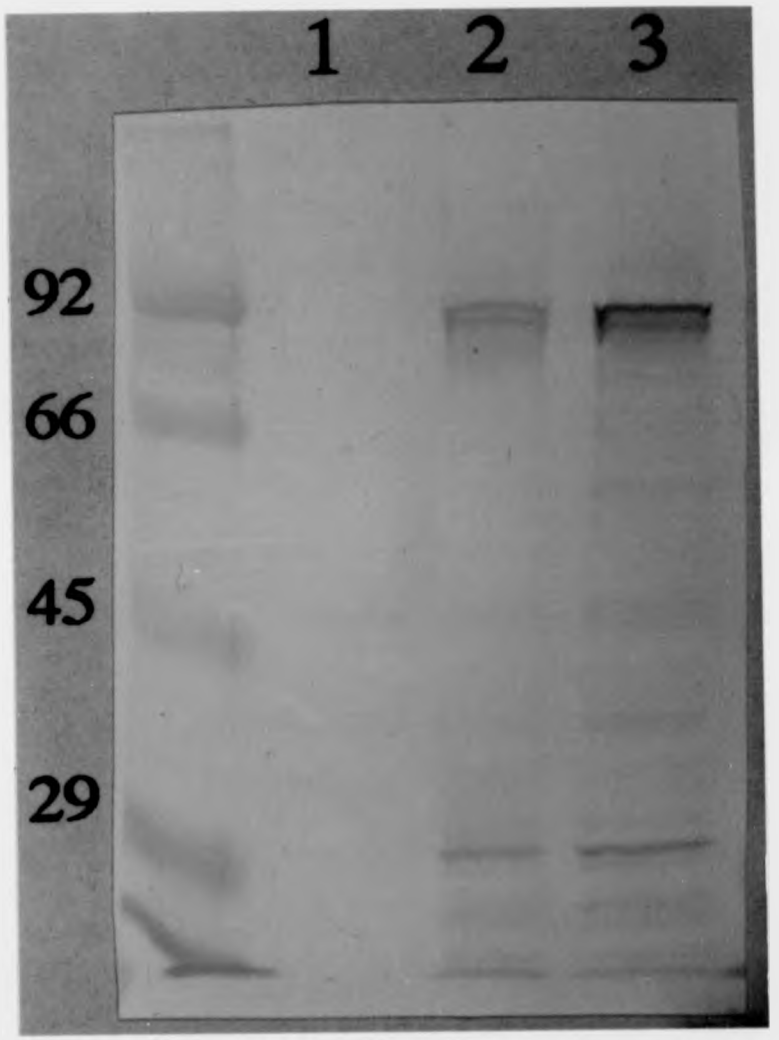


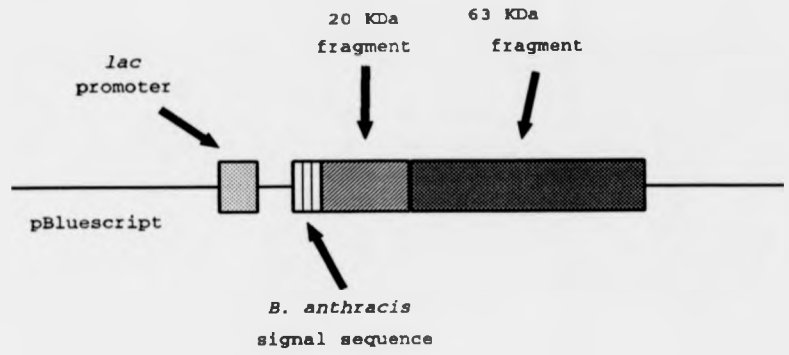
Figure 4.5 Western blot comparing PA expression from pPA26 and pCB1

Figure 4.6 Phagemid pORF1

A. This shows the regulatory and coding regions for pORF1. It is the same as pCB1 except that the promoter for the PA ORF is now the *E. coli lac* promoter from pBluescript.

B. This shows the DNA insert cloned into pBluescript II. PCR primers were designed to allow amplification of the PA ORF from the ribosome binding site (RBS) to beyond the C-terminal end of the PA ORF. See Figure 4.7. The PCR product was restricted to leave *Kpn*I and *Xba*I ends. This was cloned into *Kpn*I-*Xba*I cut pBluescript.

A.



B.

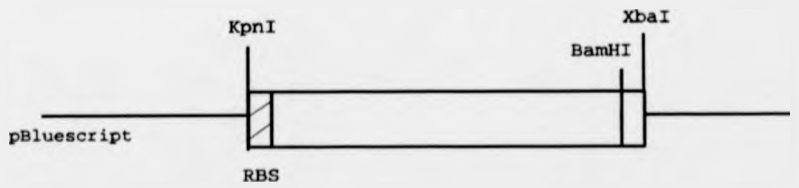


Figure 4.6 Phagemid pORF1

Figure 4.7 PCR primers for pORF1

A. This shows the *Hin*dIII-*Bam*HI insert from pPA26 which contains the PA gene. PCR primers PAALL5 and PAALL3 were designed to allow amplification and cloning of the Ribosome binding site (RBS) and PA ORF into pBluescript after the *lac* promoter. Primer PAALL5 has an homologous region from the start point of the PA mRNA transcript and includes the RBS of the PA gene and a 'tail' which contains the sequence restricted by *Kpn*I. the primer PAALL3 has an homologous region to the 3' end of the insert, beyond the stop codons of the PA gene. It also contains a 'tail' with a unique sequence for the restriction enzyme *Xba*I. During PCR amplification the 'tails' will become incorporated into the product.

B. The PCR product was restricted with *Kpn*I and *Xba*I and cloned into similarly cut pBluescript II KS+. Phagemid pORF1 contains the PA ORF transcribed from the *lac* promoter.

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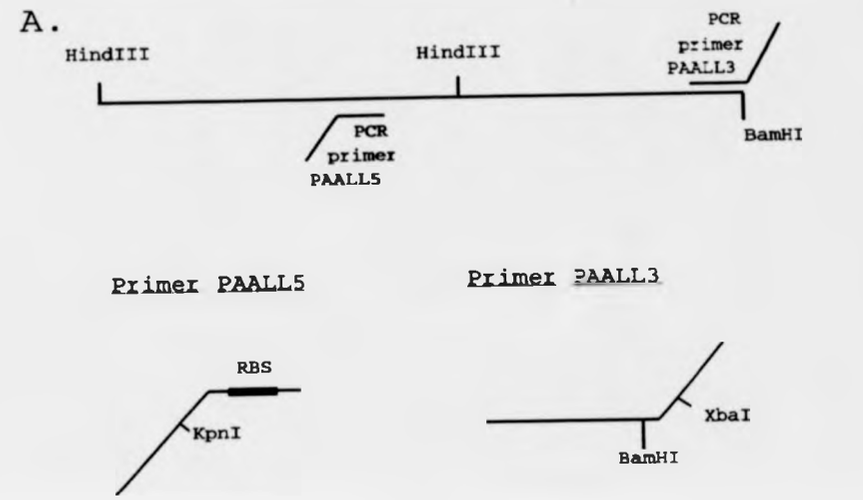

Figure 4.7 PCR primers for pORF1

was designed to bind at the start point of the PA mRNA transcript and also had in its tail a stop codon in frame with the β-galactosidase ORF of pBluescript. Although the *Bam*HI site downstream of the gene could have been used, a *Xba*I site was included in PAALL3 (the downstream primer) to allow insertion in the same manner into pUC plasmids, if required.

The primers were used in a standard PCR reaction with CsCl purified pPA26 as the template and cycles of 94°C for 3 mins, 25 cycles of 94°C for 30 secs - 37°C for 30 secs - 72°C for 90 secs, 72°C for 10 mins and refrigeration until required. Four 100 μl reaction tubes were cycled and the combined products analyzed by agarose gel electrophoresis, the appropriate band was excised and Qiaex purified. The PCR product was then digested with *Kpn*I and *Xba*I restriction endonucleases. pBluescript II KS+ was also digested with *Kpn*I and *Xba*I enzymes and gel purified. The vector and insert were ligated and electroporated into *E. coli* JM 101. Clones containing the insert were identified using 2 PCR primer sets. One contained primers T3 and T7 which bound outside of the polylinker. These show the size of the inserted DNA. The other PCR primer set contained primer T7 and, what was originally designed as a sequencing primer, PAS7R which binds within the insert and will only produce a product with T7 if the insert DNA is in the correct orientation. The results obtained with these primer sets are shown in Figure 4.8. Whole cell lysates from induced cultures of the four clones were Western blotted with mouse MAb and the clones which were positive with PCR showed expression of full-length PA. One clone was designated pORF1. A large scale Qiagen plasmid preparation was made and it restricted with a panel of enzymes as expected. Large amounts of ssDNA could not be recovered following co-infection with a helper phage. Therefore ssDNA was prepared using the Dynabead method with primers Biotinylated-T3 and T7. The product was sequenced from PAS14 and the sequence at the junction of the polylinker and PAALL5 verified.

4.3.2 PA expression from pORF1

Initially the expression of PA from pORF1 was investigated in *E. coli* and then subsequently in *S. typhimurium*.

E. coli clones containing pBluescript (as a negative control), pPA26, pCB1 and pORF1 were grown overnight in L-broth supplemented with ampicillin. IPTG was added to the *E. coli* / pORF1 culture, to induce expression from the *lac* promoter, and incubation continued for 1 hour. 10 ml of the cultures were centrifuged and 1xLB added to the pellet

Figure 4.8 PCR to identify pORF1 clones

Four colonies were picked from a *E. coli* JM 101 ligation plate and mixed into 100 μl of water in a PCR tube and also streaked onto a fresh plate to preserve the clone. The PCR tube was heated for 3 mins in a boiling water bath to lyse the cells and then the 100 μl was split into two 50 μl samples. One aliquot was amplified with 50 μl of a master mix containing primers T3 and T7 which bind outside the polylinker. These show the size of the inserted DNA. If the insert is present these primers would be expected to give a band of 2.55 kbp. The other 50 μl aliquot was amplified with T7 and what was originally designed as a sequencing primer, PAS7R, which binds within the insert and will only produce a product (1.52 kbp) with T7 if the insert DNA is in the correct orientation. 10 μl of the reaction mixes were run on a 0.7% agarose gel. The photograph shows that colonies 1, 3 and 4 give the expected pattern.

Three MW bands are labelled to allow size comparisons.

Lanes, from the left

Lane 1	BCL DNA MW markers III
Lane 2	Colony 1, T3 and T7
Lane 3	Colony 1, T7 and PAS7R
Lane 4	Colony 2, T3 and T7
Lane 5	Colony 2, T7 and PAS7R
Lane 6	Colony 3, T3 and T7
Lane 7	Colony 3, T7 and PAS7R
Lane 8	Colony 4, T3 and T7
Lane 9	Colony 4, T7 and PAS7R
Lane 10	Negative control, T3 and T7
Lane 11	Negative control, T7 and PAS7R

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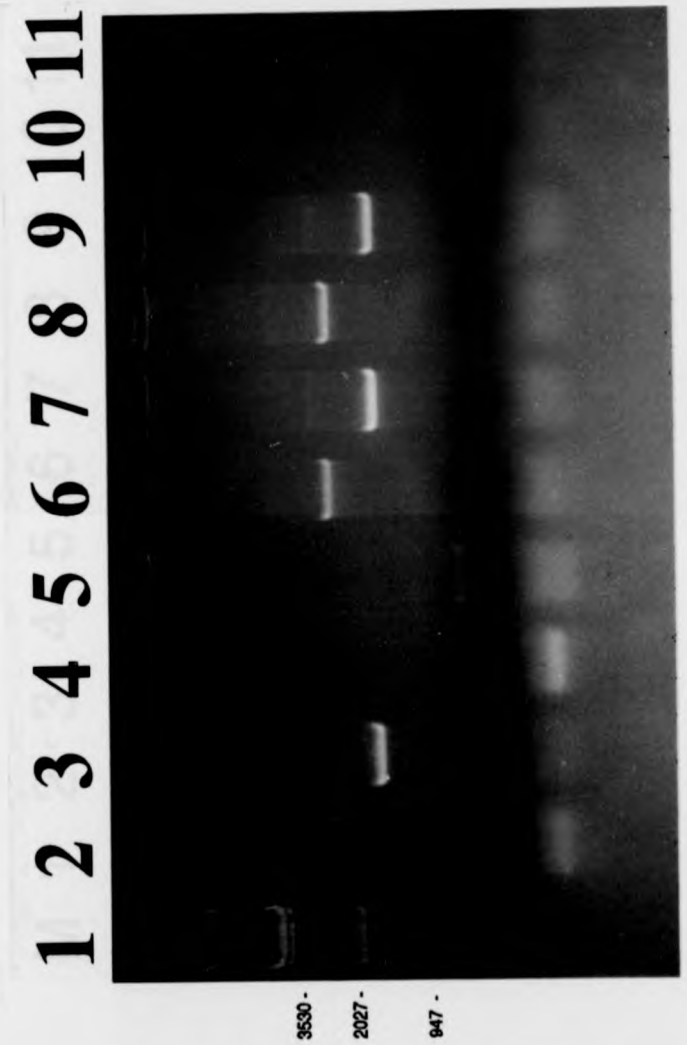

Figure 4.8 PCR to identify pORF1 clones

to give equal cell numbers / ml (based on Abs_{600} readings). A fraction of the whole cell lysates equivalent to 1.5×10^8 cells was analyzed by SDS-PAGE and Western blotted. This is shown in the top half of Figure 4.9. Under these experimental conditions, PA expression from pPA26 and pCB1 was too low to be visualised without chloramphenicol amplification even though 50 times more cells were loaded in each lane than in Figure 4.5. Expression of PA from pORF1 was visible on this Western blot showing that altering the promoter sequence increased the level of protein expression. The double banded appearance seen in chloramphenicol amplified lysates from pPA26 and pCB1 (Figure 4.5) is easily seen in *E. coli* / pORF1. In addition, several breakdown products were visible.

Altering the promoter sequence has therefore increased the expression of PA in *E. coli* but it was necessary to see if this also applied, as expected, in *S. typhimurium*. To investigate this, plasmids were recovered from *E. coli* and electroporated into *S. typhimurium* LB 5010. The modified plasmid was recovered and electroporated into *S. typhimurium* SL 3261. Clones containing the plasmids were identified and cultured as above with cell numbers in the lysates adjusted to match the *E. coli* lysates (1.5×10^8). As *S. typhimurium* SL 3261 does not contain the *lacI*^{q} gene the *lac* promoter in pBluescript would not be repressed and so IPTG induction was not required. Expression of PA from these clones is shown in Figure 4.9. A similar banding pattern to *E. coli* was seen although the bands produced by the *S. typhimurium* / pORF1 clone appear less dense. To investigate if all the bacteria were still ampicillin resistant (Amp^r) at the end of the incubation period. 10^{-5} dilutions of the *S. typhimurium* cultures were plated out on both L-agar and L-agar supplemented with ampicillin. The same number of colonies grew on both plates for all cultures except the *S. typhimurium* /pORF1 clones where only 80% of the colonies were Amp^r.

4.4 mRNA secondary structure predictions

In changing the promoter from the *B. anthracis* PA promoter in pPA26 to the *E. coli* *lac* promoter in pORF1, the mRNA primary sequence has been changed. The PA mRNA is preceded by a short region of the β-galactosidase mRNA in pORF1. This would be expected to lead to a different mRNA secondary structure. mRNA secondary structure can influence expression. The secondary structure of the mRNA can be predicted using the algorithm of Zuker & Stiegler (1981) which is implemented within the computer programme

Figure 4.9 Western blot comparing PA expression from pPA26, pCB1 and pORF1

Whole cell lysates, adjusted for cell number, were prepared from cultures of *E. coli* / pBluescript, pPA26, pCB1 and pORF1. The *E. coli* / pORF1 culture was induced with IPTG for 1 hour prior to harvesting the cells. *S. typhimurium* SL 3261 containing the same plasmids were grown similarly but without IPTG induction of *S. typhimurium* / pORF1 (as expression is constitutive). The pellet from 10 ml of each culture was suspended in 660 to 1240 μl of 1xLB (depending on Abs_{600} of the culture) and boiled for 3 mins. This lysate was diluted 1:5 in 1xLB and 20 μl loaded in each well. This is equivalent to the protein from approximately 1.5 x 10^8 cells loaded in each lane. Bio-Rad prestained MW markers were run in the left lane and apparent MWs are shown. The gels were blotted onto PVDF membrane, blocked in Blotto, and incubated in a primary antibody of mouse anti-PA monoclonal antibodies, and a secondary HRP-conjugated anti-mouse IgG. The substrate used to visualised the bands was DAB.

E. coli lysates are shown in the top blot, *S. typhimurium* in the bottom.

Lane 1 pBluescript

Lane 2 pPA26

Lane 3 pCB1

Lane 4 pORF1

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Figure 4.9 Western blot comparing PA expression from pPA26, pCB1 and pORF1

Genmon.

The predicted secondary structures for the first 100 nt of the mRNA produced by pPA26 (and pCB1) and the first 150 nt of the mRNA produced by pORF1 are shown in Figure 4.10. For pPA26 the free energy of this region is -3.4 kcal with 3/3 bases of the AUG start codon and 0/4 of the RBS AGGA involved in base-pairing. For the 150 nt folded in pORF1 the free energy is -13.6 kcal with 0/3 and 3/4 bases of the PA gene's AUG and AGGA involved in base-pairing.

4.5 Conclusions

PA expression from the PA gene, driven by the *B. anthracis* PA promoter, can only be detected by Western blotting under special conditions. A candidate vaccine strain should express the heterologous protein at levels approaching 5% of the cell protein producing a band which should be easily visualised on a Coomassie stained gel. The experimental data presented here suggests that plasmid copy number can affect expression but only in proportion to the plasmid number. However, the amount expressed in *E. coli* using the native *B. anthracis* sequence is still small. This may be due to a sub-optimal regulatory region and so the effects of altering the promoter sequence to one active in *E. coli* were evaluated. Replacing the *B. anthracis* PA promoter with the *E. coli lac* promoter did increase expression from undetectable levels on a standard whole cell lysate Western blot to an easily detectable band. When expressed in *Salmonella*, the intensity of the PA band on a Western blot was less than in *E. coli*. An explanation for the weaker bands is that the plasmid is less stable in *S. typhimurium* than *E. coli* and that a proportion of the bacteria have lost the plasmid and are not expressing PA. This situation would arise if the β-lactamase produced by the bacteria depletes the ampicillin present in the media allowing survival of plasmid-free ampicillin sensitive (Amp^s) bacteria. It was shown that pORF1 was lost from a proportion of the bacteria. The stability of the phagemids and the time-course of expression is further investigated in Chapter 7.

The level of expression of a protein is determined by the rate of transcription, stability of the mRNA and efficiency of translation. In this chapter the effect of altering the promoter has been investigated. This may also have indirectly affected interactions at the RBS and also the stability of the mRNA. In order to try and explain the experimental results, a comparison of the promoters, RBSs and mRNA secondary structures was made.

Figure 4.10 Predicted secondary mRNA structures

mRNA secondary structures were predicted using the algorithm of Zuker & Stiegler (1981) implemented within the Genmon suite of programmes on a VAX computer.

A. Predicted structure for the first 100 nt of the mRNA of pPA26. The AGGA region of the PA S/D region is underlined. The AUG start codon and direction of translation for PA are shown by an arrow.

B. Predicted structure for the first 150 nt of pORF1. This region is shown in Figure 4.11. 150 nt are folded as the PA RBS is located downstream of the β-galactosidase (β-gal) fragment ORF on the mRNA. The AGGA of the S/D regions of PA and β-gal are underlined and marked. The AUG start codon and direction of translation for PA are shown by a wide arrow; the AUG for β-gal with a narrow arrow.

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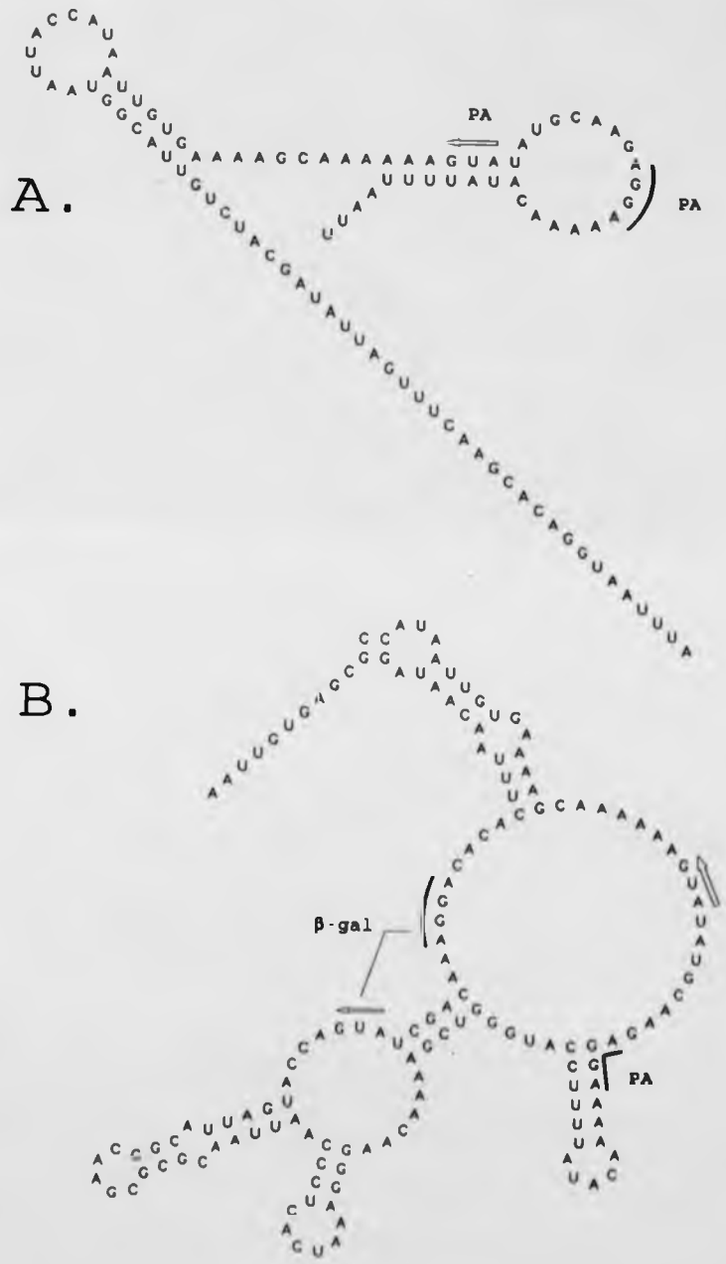

Figure 4.10 Predicted secondary mRNA structures

The consensus sequences of *Bacillus* and *Salmonella* promoters are known to differ and so it would be expected that altering the promoter sequence to one known to be active in *Salmonella* would increase expression of PA, although other factors could be limiting the level of expression. It is generally accepted that *E. coli* RNA polymerase can utilize many Gram positive promoters efficiently, although the reverse is not true. Many Gram positive genes have been expressed in *E. coli* (Graves & Rabinowitz, 1986). The consensus sequences for the *E. coli* $E\sigma^{70}$ promoters, *B. subtilis* $E\sigma^{43}$ promoter and the sequences of the promoters in pPA26 and pORF1 are shown in Table 4.1, A and B. The regions shown are the main areas of homology but other areas, such as an AT rich area around -43, are also important. The *E. coli* consensus sequence was derived from 168 promoter sequences. It was not optimised for high level expression promoters. The *lac* promoter is a common promoter in cloning vectors as it produces good levels of expression and it can be regulated (Winnacker, 1987). Expression from this promoter can be increased by specific mutations (such as UV5 which alters the *lac* -10 box to the consensus TATAAT) and the construction of hybrid promoters such as *tac*. Apart from the actual sequence of the -10 and -35 regions, the spacing between these regions is important. The spacing in the *lac* hybrid promoter *tac* is optimal at 16 bp. Increasing the spacing in *tac* to 17 or 18 bp reduces expression to 90% and 65% respectively (Brosius *et al.*, 1985). The comparison shows that the PA promoter has an identical -10 box to both the *E. coli* and *B. subtilis* consensus sequences and only one base different in the -35 region. The PA promoter -10 and -35 regions therefore have better homology with the consensus sequences than the *lac* promoter, yet when the PA promoter was replaced with the *lac* promoter, expression was increased. The spacing between the -10 and -35 regions is greater in the PA promoter than the consensus sequences (20 verses 16-18 bp). As this spacing has been shown to have a large effect on expression from the *tac* promoter, the 20 bp spacing may be the reason for the lower expression from the PA promoter compared to the *lac* promoter. This would have to be shown experimentally by deleting up to 4 bases from the region between the -10 and -35 boxes of the PA promoter.

The RBS has been defined as the area containing the Shine/Dalgarno (S/D) region and the AUG start codon, but the term is also used for the longer region protected by the ribosome during binding. The term 'translation initiation region' (TIR) defines a similar region although some authors use it to also encompass more distant regions of the mRNA

Table 4.1 Comparison of promoter and RBS sequences

A This shows the sequences for the -35 and -10 boxes of four promoter regions with the spacing between them. The promoter consensus sequences for *E. coli* is from Winnacker (1987) and for *B. subtilis* is from Moran *et al.* (1982). The PA promoter sequence is from Welkos *et al.*, (1988) and the sequence of the *lac* promoter from pBluescript (Stratagene Ltd).

B This shows the sequences of the promoters aligned on the -10 box. The *E. coli* consensus sequence is from Winnacker (1987), with '-' representing bases with little consensus and small letters where homologies are less than 70%. The *B. subtilis* consensus sequence is from Graves and Rabinowitz, (1986), with small letters for bases with more than 41% homology, capital letters for homologies above 50% and bold letters for homologies above 75%. For comparison, the sequences for the PA and *lac* promoters are shown as well. All sequences end at the -1 position.

C This shows the sequence at the 3' end of the *E. coli* 16SrRNA (Shine & Dalgarno, 1974) and its compliment aligned with the consensus region of the *E. coli* RBS (from Scherer *et al.*, 1980) and the RBS of PA and lacZ in pORF1. The last three sequences are aligned on the AUG start codon. For purposes of orientation, the AGGA motif in the RBS is underlined.

A

Promoter	-35 box	spacing (bp)	-10 box
E. coli $E\sigma^{70}$	TTGACA	16-18	TATAAT
B. subtilis $E\sigma^{43}$	TTGACA	17-18	TATAAT
PA in pPA26	TTGAAA	20	TATAAT
lac in pORF1	TTTACA	18	TATGTT

B

	Promoter sequence aligned on -10 box
E. coli	----------TTGaca-ttt (6-9) attttgtTATAATg(4-7)c
B. subtilis	-Ta-AAAAA----TTGAcA---a--A---a-T-TG-TATAATAAtAt--
PA	TTATATTATATTGAAACTAAAGTTTATTAATTTCAATATAATATAAAT
lac	GGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGG

C

	RBS sequences aligned on start codon
3' end 16SrRNA	AUUCCUCCACUAG UAAGGAGGUGAUC - compliment
E. coli	UU.UUAAAAAUUAAGGAGGUAUAUUAUGAAAAAAAUUAAAAAACUCAA
PA	AUUUUAUACAAAAAGGAGAACGUAUAUGAAAAAACGAAAAGUGUUAAU
lacZ	UAACAAUUUCACACAGGAAACAGCTAUGACCAUGAUUACGCCAAGCGC ↑ +1

Table 4.1 Comparison of promoter and RBS sequences

that can affect translation. In this discussion, RBS is used to mean the region from the S/D sequence to the start codon, and the TIR to include regions up- and down-stream of the RBS that affect initiation of translation. The efficiency of translation is affected by the primary and secondary structure of the mRNA in this region. This region has been characterised extensively by both an analysis of the nucleotide sequences in many different TIRs (Stormo *et al.*, 1982) and by the effect of alterations in this site on expression (Curry & Tomich, 1988). The S/D region sequence is complimentary to the 3' end of the 16S ribosomal RNA and is usually 5-10 nt from the start codon. In addition, the codons either side of the start codon can affect translational efficiency (Curry & Tomich, 1988; Looman *et al.*, 1987) as can the primary sequence at the 5' end of the ORF.

pORF1 was constructed to translate the PA gene using the PA RBS, but with the *lac* promoter controlling transcription, so that a comparison could be made between this construct and pPA26. The nucleotide sequences of the PA gene RBSs in pPA26 and pORF1 are therefore identical. pORF1 also contains the *lacZ* RBS upstream of the PA RBS. These RBSs can be compared to the *E. coli* consensus RBS sequence and 3' end of the 16S rRNA sequence. This is shown in Table 4.1, C. The *E. coli* consensus sequence was obtained from a comparison of 68 sequences and does not relate to highly expressed genes. It can be seen that the PA RBS is similar to the *E. coli* consensus sequence and it does meet all of the rules proposed by Scherer *et al.* (1980) and Stormo *et al.* (1982) except that the PA RBS does not have an A or T at position +10 or +11. This small change may reflect the different codon usage of *Bacillus* genes. Although the PA RBS should function adequately in *E. coli*, the sequence may not be optimal. The *lacZ* RBS also shows a significant variation from the consensus sequence.

Subsequent to the construction of pORF1, it was realised that the *lacZ* RBS upstream of the PA coding region might affect expression of PA. Although the ORF for β-galactosidase is terminated in the region of the AUG initiation codon of PA, and is out of frame with the coding region of PA, it is possible that the ribosome may terminate translation of β-galactosidase but remain bound to the mRNA. It may then recognise the PA RBS, shift frame, and translate the PA open reading frame. This region is shown in Figure 4.11. This effect has been described for the two cistron expression of Bovine Growth Hormone (Schoner *et al.*, 1984). The effect of this would be to raise the apparent translation initiation efficiency of the PA gene.

Figure 4.11 Nucleotide sequence of TIR of pORF1

The nucleotide sequence from the *lac* promoter to the ORF of PA is shown. The -10 and -35 regions of the promoter are underlined and marked beginning at 2096 and 2072 respectively. The proposed transcription start is marked in bold at 2108. The β-galactosidase and PA RBSs are marked. The amino acid sequence coded for by the ORFs is shown below the nucleotide sequence using the single letter amino acid code. The amino acid sequence for β-galactosidase is shown in lowercase letters. It is terminated at a TGA stop codon which overlaps the ATG start codon for the PA ORF. The amino acid sequence for PA is shown in uppercase letters.

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```
                              lac promoter
                         -35                        -10
CACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGT
----.----+----.----+----.----+----.----+----.----+  2100
GTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACA

      ---------->                    β-galactosidase RBS
      start of mRNA                S/D       Start codon
TGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGAC
----.----+----.----+----.----+----.----+----.----+  2150
ACACACCTTAACACTCGCCTATTGTTAAAGTGTGTCCTTTGTCGATACTG

                        β-GALACTOSIDASE >>m  t

CATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCT
----.----+----.----+----.----+----.----+----.----+  2200
GTACTAATGCGGTTCGCGCGTTAATTGGGAGTGATTTCCCTTGTTTTCGA

 m  i  t  p  s  a  q  l  t  l  t  k  g  n  k  s  s

                          PA RBS
                      S/D        Start codon
GGGTACCTTTTATACAAAAAGGAGAACGTATATGAAAAAACGAAAAGTGT
----.----+----.----+----.----+----.----+----.-----  2500
CCCATGGAAAATATGTTTTTCCTCTTGCATATACTTTTTTGCTTTTCACA

  v  p  f  i  q  k  g  e  r  i  .

                          PA >>M  K  K  R  K  V  L
```

Figure 4.11 Nucleotide sequence of TIR of pORF1

Thus, the PA RBS should initiate translation quite effectively. There is no ideal RBS, due to the interactions which occur with other regions of the gene (Curry & Tomich, 1988), and changes would have to be made and tested experimentally to arrive at a better sequence.

Although the primary sequence is identical for the RBS of pPA26, pCB1 and pORF1, the secondary structure may vary. mRNA secondary structure around the RBS is considered important in regulating the initiation of translation (Schoner *et al.*, 1984; Schauder & McCarthy, 1989). Secondary structure may also affect the stability of the mRNA. The structure of the mRNA was predicted using the algorithm of Zuker & Stiegler (1981) which was implemented within the computer programme Genmon. This found a conformation of minimum free energy for the RNA molecule based on published values of stacking and destabilising energies. The structures produced represent one possible configuration with the lowest free energy; but totally different structures, with free energies close to the minimum, may be more biologically significant. The programme produced a structure without weighting the increased likelihood of base-pairing occurring locally rather than between distant regions of the RNA molecule. The predicted structure for any region will therefore vary depending on the total length of the mRNA presented for folding. As the mRNA is synthesised, the 5' end is more likely to become involved in local interactions. One method of using the prediction programme is to fold progressively more of the 5' end of the mRNA and look for stable secondary structure features involving the RBS (Schoner *et al.*, 1984).

The information from secondary structure predictions has been correlated with expression in two ways. Firstly, the free energy of the predicted structure has been shown to correlate with translational initiation efficiency (Schauder & McCarthy, 1989). Secondly, the masking or exposing of the S/D region and start codon within regions of secondary structure may also affect expression (Schoner *et al.*, 1984; Winnacker, 1987). Although one of these approaches may explain many results of expression studies, some authors have found exceptions that cannot be explained (Looman *et al.*, 1987). This failure is probably due to the complexity of the interactions between primary sequence changes and secondary structure predictions.

The free energies of the predicted TIR structures (-3.4 kcal for pPA26 and -13.6 kcal for pORF1) do not support the observed expression results as pORF1 expresses more PA

than pPA26 and yet has the more stable structure. The involvement of the initiation codon in base-pairing in pPA26 could limit expression, as could the involvement of the AGGA of the PA RBS in pORF1. The AUG and AGGA for the β-galactosidase RBS are not involved in base-pairing and it is possible that a ribosome translating the β-galactosidase ORF could breakdown the secondary structure involving the PA RBS and so aid translation of this second cistron (Schoner *et al.*, 1984). This could be partly responsible for the increased expression of PA from pORF1.

Although predictions of secondary structure can produce direct correlations with expression in tightly controlled studies, the complex interactions of changes in the primary sequence of the TIR make it difficult to predict the overall effect of changes designed to produce favourable secondary structural changes.

From the above it can be seen that the PA promoter and RBS should function in *E. coli*, but the level of expression is disappointing. Altering the promoter to the *E. coli lac* promoter increased expression (pORF1). The level of expression is known to be a complex interaction of many factors. Although experimental evidence has shown particular features which can affect expression, a small change can have many potential effects. A point mutation in the first few codons might alter the affinity of the ribosome for the RBS, the mRNA secondary structure and the codon usage. It is therefore not possible to make specific and authoritative predictions concerning alterations to the PA genes promoter and TIR which would increase the level of expression. Any changes would have to be investigated experimentally and would not be certain to lead to increased expression.

In conclusion, PA expression can be increased by altering the gene copy number but this only produces a proportional increase to the change in copy number. Changing the promoter from the *B. anthracis* PA promoter to the *E. coli lac* promoter had a significant effect on expression. When this change was analyzed at the DNA sequence level several possible reasons for this effect were identified but their contribution to expression would need to be experimentally assessed. Further improvements to expression should be possible, but no obvious limiting sequences were identified in this analysis. Other factors may be limiting expression.

CHAPTER 5

Rational approach to increasing expression of PA

5.1 Introduction

As discussed in Chapter 4, increased production of PA in *S. typhimurium* would probably improve the potential of a putative vaccine strain. It has been shown that increasing the copy number of the PA gene (pPA26 to pCB1) had a small effect on expression, but altering the promoter from the PA promoter to the *lac* promoter (pCB1 to pORF1) had a larger effect on expression. The level of expression of a gene is determined primarily by three factors: the rate of transcription, the stability of the mRNA, and the efficiency of translation. The amount of protein present in a cell is also related to the stability of the protein once it is produced. There are various strategies to affect each of these factors so as to maximise the protein present in the cell. These include modifications of the promoter sequence and ribosome binding region, changes affecting mRNA secondary structure, alterations of the codon usage and modifications to the protein conformation and cellular location. Most of the experimental work related to the level of protein expression has been carried out in *E. coli*. It is considered that in general the same effects will usually occur in the closely related *S. typhimurium*. As discussed in Chapter 4, the *lac* promoter, PA RBS and mRNA secondary structure should be adequate for initiating expression of PA. It is therefore possible that the efficiency of translation could be limiting expression, possibly due to codon usage differences. This factor was considered to see if any obvious changes could be made to increase expression of PA further. From all the available information a strategy to increase expression was selected and constructs made to evaluate this.

5.1.1 Codon usage

Codon usage within the ORF of a gene has been reported to affect expression (Sharp & Li, 1986). Although the nucleotide sequence of the N-terminal amino acids can have a large effect on expression by affecting the RBS and mRNA structure (Looman *et al.*, 1987; Barnes *et al.*, 1991), this is considered in section 4.5. In this section, the codon usage of the PA ORF as a whole will be considered.

The usage of codons is non-random and varies from organism to organism (Makoff *et al.*, 1989; Andersson & Kurland, 1990). It is also known that there is a positive correlation between the degree of codon bias and level of expression within *E. coli*. It appears that this forms a means of regulation of expression within *E. coli* (Sharp & Li, 1986).

It has been shown that *B. subtilis* has an unbiased codon usage compared to *E. coli* (Ogasawara, 1985) and *S. typhimurium* (Wada *et al.*, 1992). The codon usage for *E. coli*, *S. typhimurium*, *Bacillus* species and PA is shown in Table 5.1. From this data the frequency of amino acid use was calculated. This showed that *E. coli* and *S. typhimurium* have similar amino acid use which differs to *Bacillus* species. PA amino acid use is more similar to *Bacillus* species but does have some major differences. For instance, asparagine (ASN) occurs at a frequency of 90 per 1000 amino acids in PA but only 54 per 1000 in *Bacillus* codons. This is displayed graphically in Figure 5.1.

For expression studies a comparison of the use of synonymous codons is more valuable. From the data shown in Table 5.1 it was concluded that *E. coli* and *S. typhimurium* have similar codon usage but this differs, markedly in some cases (eg LEU), from *Bacillus* and PA codon usage. A comparison of codon usage for three amino acids is shown in Figure 5.2. Although *Bacillus* codon use was different to *E. coli* and *S. typhimurium*, codon usage within the PA gene was even more diverse. An analysis of codon usage in *E. coli* (Grosjean & Fiers, 1982) and *S. typhimurium* (Sharp, 1991) showed that different codons are used in strongly and weakly expressed genes (possibly related to the strength of codon-anticodon interaction) and codons which are predominately found in weakly expressed genes can be identified. Other codons within a synonymous set may occur rarely in highly expressed genes of *E. coli* and this may be correlated to tRNA pools (Sharp & Li, 1986). Several theories concerning the mechanism of codon usage affecting level of expression have been suggested. One theory favours the selection of rare codons to limit expression. Codon usage might affect expression when tRNA pools become limiting or the interaction between the codon and anticodon is either too strong or too weak. Thus even if the level of mRNA is increased, unfavourable codon usage may limit expression. Rare codons may cause ribosome stalling which could expose regions of the mRNA to endonucleases and so alter the mRNA turnover (Makoff *et al.*, 1989). An alternative view suggests that the occurrence of rare codons in regulatory and weakly expressed genes results from the absence of strong negative selection rather than from positive selection as a means of regulation of expression (Andersson & Kurland, 1990). The same authors conclude that the codons preferred by strongly expressed genes are positively selected to optimise growth rates in good culture conditions by allowing a smaller subset of tRNAs to predominate. The ratios of isoacceptor tRNA levels do change as growth rates increase to favour the codons

Table 5.1 Codon Usage

The percentage codon usage for each amino acid is shown on the left side of the table.

The codon use per 1000 codons is shown on the right side of the table.

Data for *E. coli* (E.c), *S. typhimurium* (S.t) and *Bacillus* (Bac) are derived form Wada *et al.*, (1992). The data for PA (PA) was obtained using the PA sequence from Welkos *et al.*, (1988) and the TRANS programme from DNAStar.

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Amino Acid	Codon	percent codon usage				Codons per 1000			
		E.c	S.t	Bac	PA	E.c	S.t	Bac	PA
ARG	CGA	3.4	4.1	11.8	10.3	3.1	3.5	5.1	3.9
	CGC	38.5	39.3	18.5	0	22.1	22.5	8.1	0
	CGG	8	10.8	10	13.8	4.6	6.2	4.4	5.2
	CGU	42	34.8	23	10.3	24.1	19.9	10.1	3.9
	AGA	3.7	5.4	28.7	55.2	2.1	3.1	12.6	20.9
	AGG	2.4	3.5	8.2	10.3	1.4	2	3.6	3.9
LEU	CUA	3.2	4.6	8.2	4.8	3.2	4.6	7.1	3.9
	CUC	9.9	9.9	8.8	3.2	9.9	9.9	7.6	2.6
	CUG	54.4	49.6	16.3	1.6	54.6	49.4	14.1	1.3
	CUU	10.2	10.6	22.1	9.7	10.2	10.5	19.2	7.9
	UUA	10.9	13.5	29.8	67.7	10.9	13.4	25.8	54.9
	UUG	11.5	11.8	14.9	12.9	11.5	11.7	12.9	10.5
SER	UCA	11.9	12.5	21.1	20.8	6.8	7.4	13.7	19.6
	UCC	16.5	18.4	12.8	4.2	9.4	10.9	8.3	4
	UCG	14	14	10	9.7	8	8.3	6.5	9.1
	UCU	18.2	14.7	22.9	30.6	10.4	8.7	14.9	28.8
	AGC	26.7	27.7	17.7	2.8	15.2	16.4	11.5	2.6
	AGU	12.6	12.8	15.5	31.9	7.2	7.6	10.1	30
THR	ACA	12.1	12.4	39.8	37.9	6.5	6.8	24.1	28.7
	ACC	45.3	44.1	13.9	10.3	24.3	24.1	8.4	7.8
	ACG	23.6	28.3	24.8	19	12.7	15.5	15	14.4
	ACU	19	15.2	21.6	32.8	10.2	8.3	13.1	24.9
PRO	CCA	19.1	14.8	31.3	37.9	8.2	6.5	12.1	14.4
	CCC	10	13.7	6.7	10.3	4.3	6	2.6	3.9
	CCG	55.5	54	32.6	20.7	23.8	23.7	12.6	7.8
	CCU	15.4	17.5	29.5	31	6.6	7.7	11.4	11.8
ALA	GCA	21.8	14.7	31.2	46.3	20.6	13.5	22.1	24.8
	GCC	24.8	29	17.3	4.9	23.5	26.7	12.3	2.6
	GCG	35	40.4	23	17.1	33.1	37.2	16.3	9.2
	GCU	18.4	16	28.5	31.7	17.4	14.7	20.2	17
GLY	GGA	9.4	10.2	32.2	52.8	7	7.4	22.6	24.8
	GGC	40.5	47.2	27.1	5.6	30.2	34.3	19	2.6
	GGG	13	15.6	16.3	30.6	9.7	11.3	11.4	14.4
	GGU	37	27	24.4	11.1	27.6	19.6	17.1	5.2
VAL	GUA	16.3	17.3	28.5	39.5	11.6	12.2	19.5	22.2
	GUC	19.8	25.1	20.1	4.7	14.2	17.7	13.8	2.6
	GUG	35.5	35.2	21.8	27.8	25.3	24.8	14.9	15.7
	GUU	28.2	22.4	29.6	27.9	20.1	15.8	20.3	15.7
LYS	AAA	75.3	75.4	73	78.3	36.5	36.7	46.4	61.4
	AAG	24.7	24.6	27	21.7	12	12	17.2	17
ASN	AAC	59.5	51.3	40.3	23.2	23.9	21.3	21.7	20.9
	AAU	40.5	48.7	59.7	76.8	16.3	20.2	32.1	69.3
GLN	CAA	30.5	29.2	65.6	83.9	13.2	12.8	25.9	34
	CAG	69.5	70.8	34.4	16.1	30.1	31	13.6	6.5
HIS	CAC	48	44.1	29.8	10	10.7	9.4	6.2	1.3
	CAU	52	55.9	70.2	90	11.6	11.9	14.6	11.8
GLU	GAA	69.3	63.9	70.3	74.5	43.4	38.4	49.5	49.7
	GAG	30.7	36.1	29.7	25.5	19.2	21.7	20.9	17
ASP	GAC	40.3	38.7	32.1	12.8	21.8	21.5	17.9	7.9
	GAU	59.7	61.3	67.9	87.2	32.3	34.1	37.8	53.6
TYR	UAC	46.5	41.7	31.9	21.4	13.4	12.6	12.6	7.8
	UAU	53.5	58.3	68.1	78.6	15.4	17.6	26.5	28.8
CYS	UGC	56.5	55.8	45.2	0	4.1	6.3	3.3	0
	UGU	43.5	44.2	54.8	0	4.7	5	4	0
PHE	UUC	48.7	40.5	32.9	20.8	18.2	15.3	13.4	6.5
	UUU	51.3	59.5	67.1	79.2	19.2	22.5	27.3	24.8
ILE	AUA	7.1	11.2	16	31.6	4.1	6.7	10.8	23.5
	AUC	45.8	41.5	32.1	17.5	26.5	24.8	21.7	13
	AUU	47.1	47.2	51.9	50.9	27.2	28.2	35.1	37.9
MET	AUG	100	100	100	100	26.5	25.5	22.2	13.1
TRP	UGG	100	100	100	100	12.8	11.8	12.2	9.2
TER	UAA	66.7	66.7	63	100	2	2	1.7	1.3
	UAG	6.7	6.7	14.8	0	0.2	0.2	0.4	0
	UGA	26.7	26.7	22.2	0	0.8	0.8	0.6	0.

Table 5.1 Codon Usage

Figure 5.1 Frequency of amino acid use

Data from Table 5.1 was used to provide totals for the frequency (per 1000) that each amino acid occurs in the sequences analyzed. The totals for each amino acid are displayed for *E. coli*, *S. typhimurium*, *Bacillus* species and PA.

Note the particular differences for Ser and Asn.

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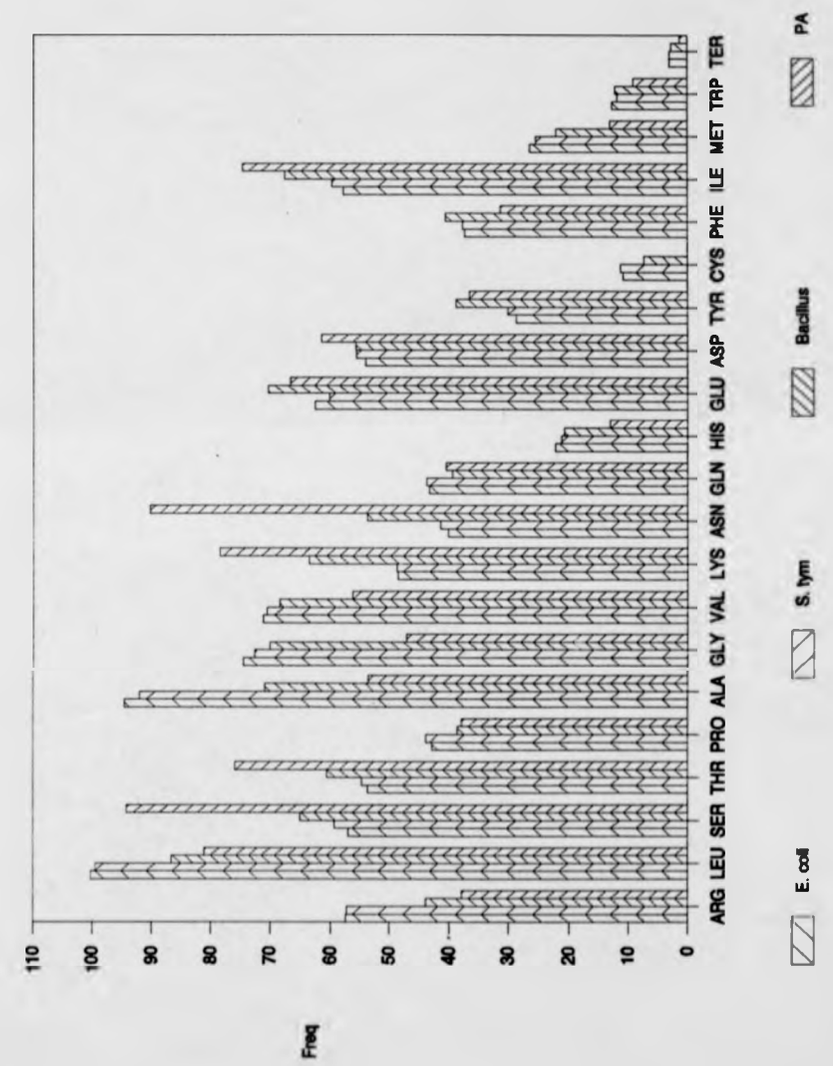

Figure 5.1 Frequency of amino acid use

Figure 5.2 Codon use for three amino acids

Data from Table 5.1 was used to compile three graphs showing the % use of each synonymous codon for three amino acids, Arg, Leu and Ser.

Note that *E. coli* and *S. typhimurium* have similar codon usage in all cases. *Bacillus* use is different to *E. coli* and *S. typhimurium* and generally shows less biased codon usage. PA codon use is particularly biased though and very different to *E. coli* , see for instance the AGA codon for Arg and the UUA codon for Leu.

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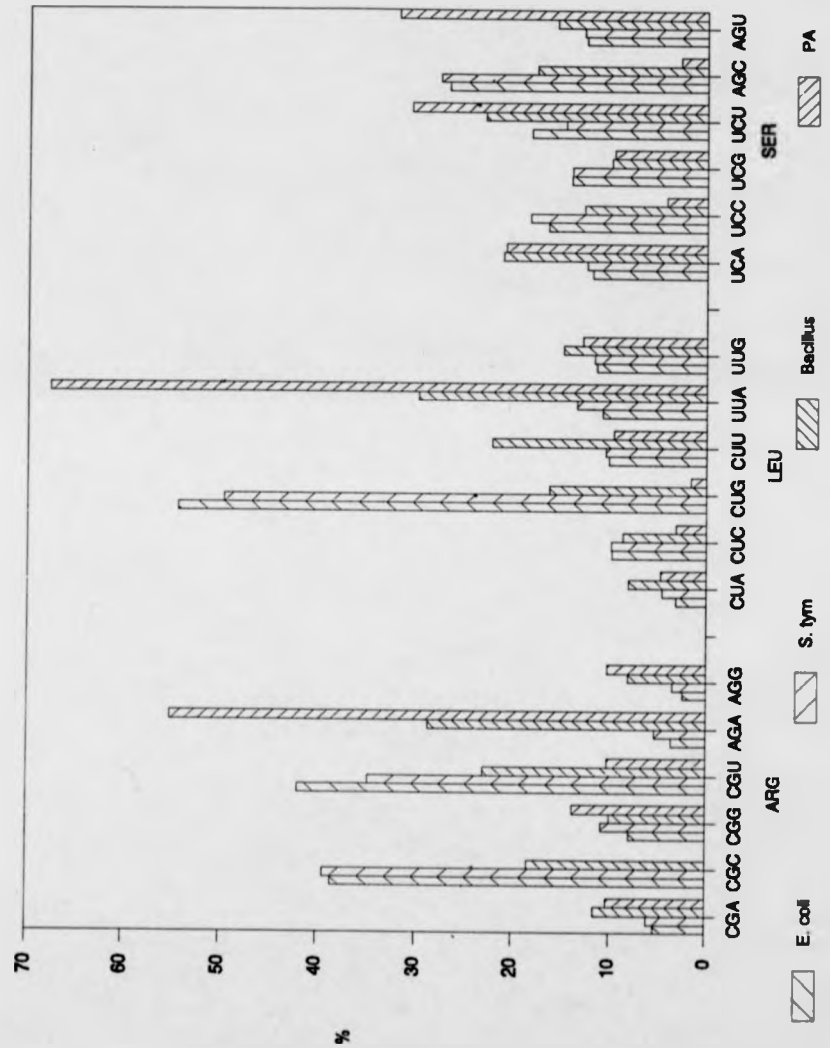

Figure 5.2 Codon use for three amino acids

found in strongly expressed genes. Organisms that show a pronounced codon usage bias, such as the enterobacteria and yeasts, are thus better adapted to periods of rapid growth (Andersson & Kurland, 1990). Possibly the less pronounced codon usage bias in *Bacillus* reflects the lack of selection pressure for rapid growth in this species.

It is possible to list codons whose use within an ORF translated in *E. coli* may adversely affect expression. Gram positive genes, with a higher A/T ratio compared to *E. coli* genes, often contain a high proportion of codons found in weakly expressed genes (termed 'weak') or codons with small tRNA pools (termed 'rare') and this is the case for the PA ORF. The use of 'rare' or 'weak' *E. coli* codons within PA is shown in Table 5.2. Of a total of 764 codons in PA, 244 (32%) can be classified as 'weak' (137 codons, 18%) or 'rare' (107 codons, 14%). The 'weak' and 'rare' codons in PA occur throughout the gene, there are no specific areas containing clusters of these codons. Although some genes with up to 10% of 'rare' codons are expressed at up to 10% of cell protein, the AT rich fragment C of tetanus toxin contains 11.7% 'rare' codons and expression was increased from 3-4% of cell protein to 11-14% by replacing the sequence with optimised codons (Makoff *et al.*, 1989). The large number of 'weak' and 'rare' codons present in PA might be expected to limit expression once mRNA levels are elevated.

Therefore the codon usage in PA is far from ideal for expression of this gene in *E. coli* (and similarly *S. typhimurium*). The scattering of 'weak' or 'rare' codons throughout the gene means that the whole ORF may have to be chemically synthesised so as to correct the codon usage. This would require the design of a cloning strategy, synthesis of complimentary oligonucleotides with optimised codon usage, annealing and cloning of the oligonucleotides, confirmation of the clone sequence, and ligation of the cloned fragments in order to recreate the ORF. The oligonucleotides could be synthesised at up to 150 bp and so at least 15 pairs would be required. This approach is impractical in this study.

5.1.2 Fusion proteins and expression of fragments

Another approach to increase the expression of heterologous proteins is to create a fusion protein of the novel protein (83 kDa PA) to the promoter, TIR and all or part of the ORF of a known highly expressed protein. The level of transcription and initiation of translation is therefore known and expression is only limited by factors such as mRNA stability and codon usage of the heterologous protein coding region. Fusion proteins may

Table 5.2 Codon replacement strategy

The table shows an analysis of the PA gene to identify the extent of the use of 'weak' or 'rare' codons. Theses codons are listed with a possible replacement codon which might improve the expression in *E. coli*. The frequency of the occurrence of these undesirable codons within the PA gene is shown.

(a) Amino acid three letter code

(b) 'Weak' or 'rare' codon in PA ORF

(c) Preferred codon to use in *E. coli* instead of codon in (b). Identified by Grosjean and Fiers (1982), on the basis of their occurrence in strongly expressed genes.

(d) Refers to the use of the 'weak'/'rare' codon (column (b)) in PA ORF. '%' is the % use of the codon amongst synonymous codons for the amino acid. 'no.' is the number of occasions that the 'weak'/'rare' codon is used in the PA ORF (total 764 amino acids). From analysis of sequence in Welkos *et al.* (1988).

(e) Reason for inclusion of codon in column (b). 'Weak' (W) codons are derived form Grosjean & Fiers (1982) and relate to the codon's occurrence in weakly expressed genes. 'Rare' codons are from Sharp & Li (1986) and are related to tRNA pool sizes.

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(a)	(b)	(c)	(d)		(e)
Amino Acid	Change from	Change to	PA %	no.	Weak or Rare
Asn	AAU	AAC	77	53	W
Arg	AGA	CGU	55	16	R
Arg	AGG	CGU	10	3	R
Ile	AUA	AUC	32	18	R
Ile	AUU	AUC	51	29	W
Pro	CCC	CCG	10	3	WR
Arg	CGA	CGU	10	3	R
Arg	CGC	CGU	0	0	W
Arg	CGG	CGU	14	34	R
Leu	CUA	CUG	5	3	R
Ala	GCC	GCU/ GCG	5	2	W
Gly	GGA	GGU	53	19	R
Gly	GGC	GGU	6	2	W
Gly	GGG	GGU	31	11	R
Tyr	UAU	UAC	79	22	W
Ser	UCG	UCU	10	7	R
Phe	UUU	UUC	79	19	W
Total				244	

Table 5.2 Codon replacement strategy

also be protected against proteolytic degradation (Enfors, 1992).

This strategy can be refined by only expressing fragments of the heterologous protein as fusion proteins. This may have benefits in that toxic proteins can be expressed as non-toxic but immunogenic fragments. Also, if codon usage is limiting, the bacteria may express proportionally more of the smaller immunogenic fragment, than the full-length construct, before tRNA pools become limiting. For this approach to work, an appropriate fragment must be identified. Epitope predictions or mapping may help identify a suitable region, or experimental approaches can be used to evaluate the immune response to expressed or chemically produce defined fragments.

5.2 Expression of 63 kDa PA

Production of PA might be increased by expressing a fragment of PA as a fusion protein. As it might be expected that conformational epitopes would be important in the generation of neutralising antibodies, the PA expressed should have the possibility of configuring in as natural a conformation as possible. One strategy that might achieve this with PA makes use of the trypsin cleavage site present between the 20 kDa and 63 kDa fragments. Protease cleavage at this site occurs *in vivo* to release the active 63 kDa C-terminal fragment which contains both the cell-surface receptor and the binding site for LF or EF (Leppla, 1991). It is known that neutralising monoclonal antibodies bind to this fragment (Little *et al.*, 1988). The 20 kDa fragment appears to play little role in immunogenicity as it is very difficult to generate monoclonal antibodies against this fragment (Little *et al.*, 1988); it may of course have other important functions. Thus the 63 kDa fragment appears to contain the important epitopes and could be expressed as a fusion protein whilst still maintaining the trypsin cleavage site between the carrier protein and the 63 kDa PA. The protein that PA is fused to may stabilise the fusion protein within the bacteria so slowing degradation and thus allowing accumulation in higher amounts. Once released from the bacteria the trypsin cleavage site may allow proteases to cleave the 63 kDa PA from the carrier protein and it may then be able to configure in a natural manner with the presentation of conformational epitopes to the immune system.

A possible advantage for this construct is that the PA signal sequence would not be present. There is a possibility that PA may be toxic due to it becoming partially integrated into the bacterial membrane during export. The strategy of removing signal sequences

and/or hydrophobic trans-membrane regions from heterologous antigens has proved useful in reducing their toxicity and stabilising expression (Curtiss *et al.*, 1990).

The vector chosen for expression of the 63 kDa form of PA was pBluescript II. This was the vector used for pORF1 and so would allow a direct comparison. The 63 kDa fragment could be expressed as a fusion with β-galactosidase from the *lac* promoter. As there are no appropriate restriction sites in the PA ORF the new construct was made using PCR and the insert was nucleotide sequenced to check for fidelity errors introduced during PCR.

A final consideration in the strategy for expression of the 63 kDa fragment of PA was based on a comparison with the experimental work on *E. coli* Heat-Labile toxin B subunit (LT-B). LT-B, like PA, is a non-toxic cell-binding protein and LT-B has been used for the targeting of fused antigens/epitopes to the immune system. Although LT-B and the related Cholera Toxin B subunit are particularly strong immunogens, if PA could be expressed stably in *S. typhimurium* it might have possibilities itself as a carrier molecule. To allow for the possibility of this, unique restriction sites were introduced at both ends of the PA insert so that subsequently sequences expressing other proteins or epitopes could be cloned onto the PA gene. The result of this modification was to alter the C-terminal end of the 63 kDa fragment by the addition of 5 aa.

In this chapter the construction of this first 63 kDa fragment phagemid and the expression of PA from it is described. Subsequently the same fragment was expressed from a different promoter (*trc*) and, as questions were raised about the possible effects of the 5 aa modifications to the C-terminal ends, constructs were made from both the *lac* and *trc* promoters with unmodified C-terminal ends. The four constructs made are shown diagrammatically in Figure 5.3.

5.3 Construction of phagemid pVET4m

pVET4m was constructed to express the 63 kDa C-terminal fragment of PA as a fusion protein with part of β-galactosidase. The phagemid pBluescript was used to express this construct from the *lac* promoter. The trypsin site was included to offer the potential for cleavage of the 63 kDa fragment from β-galactosidase. The modified C-terminal end of the fragment containing unique restriction is identified by the 'm' in the phagemid designation. The phagemid structure is illustrated in Figure 5.4.

Figure 5.3 Diagrammatical representation of the four constructs made in Chapter 5

The construct designation is shown to the left of each drawing.

pVET4m — pBluescript II KS+ based construct expressing the 63 kDa fragment of PA as a β-galactosidase fusion from the *lac* promoter. Has a modified (5 amino acid addition) C-terminus.

pVET4n — As pVET4m except has native PA C-terminus.

pNIK1n — pTrc99A based construct expressing the 63 kDa fragment of PA fused to a short linker sequence from the *trc* promoter. Native PA C-terminus.

pNIK1m — As pNIK1n except with modified (5 amino acid addition) C-terminus.

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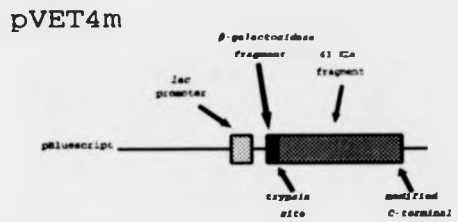

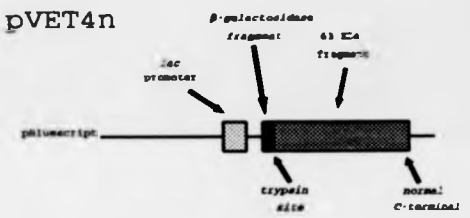

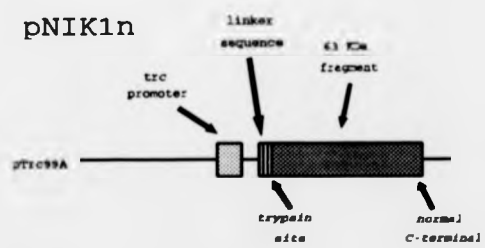

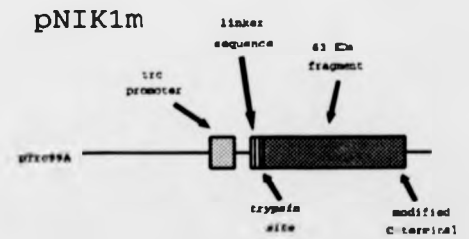

Figure 5.3 Diagrammatical representation of the four constructs made in Chapter 5

Figure 5.4 Phagemid pVET4m

A. Phagemid pVET4m was constructed to express the C-terminal 63 kDa fragment of PA fused to a short β-galactosidase fragment.

B. A blunted-ended PCR product was ligated into *Eco*RV cut pBluescript II KS+. This product created an *Eco*RV site at the N-terminal end but not at the C-terminal end. The PCR primers used contained non-homologous tails which coded for unique restriction sites at both ends. There is one internal *Pst*I site and another *Pst*I site in the pBluescript polylinker downstream of the insert. These sites were used for manipulations described in the text. The construction strategy is shown in Figure 5.5.

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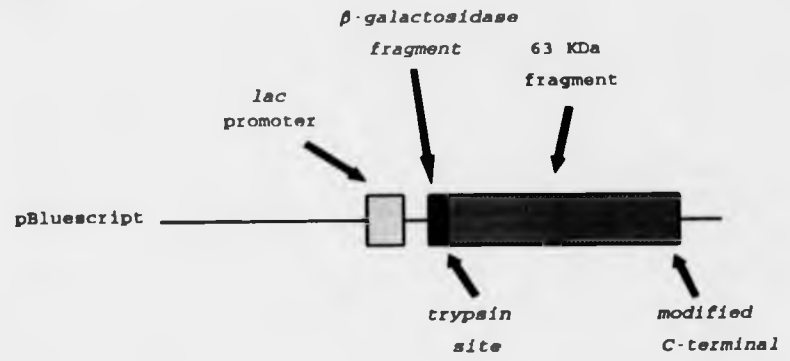
A.



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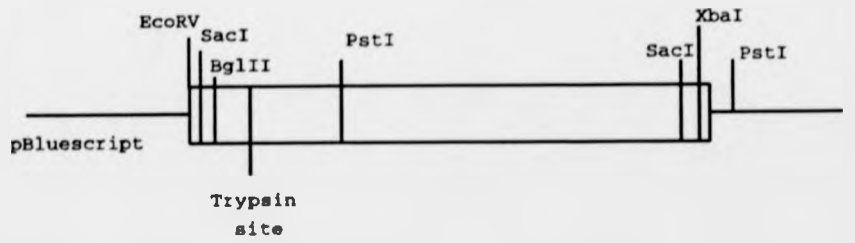

Figure 5.4 Phagemid pVET4m

The ORF in pVET4m codes for:

-34 aa from the N-terminal end of the β-galactosidase fragment in pBluescript

-a 5 aa linker coded for by the PCR primer ANTHPR3 sequence containing unique restriction enzyme sites

-3 aa from PA and then the 4 aa RKKR trypsin cleavage site and the 568 aa C-terminal 63 kDa fragment of PA (575 aa)

-5 aa addition to the C-terminal end of PA coded in the sequence of PCR primer ANTHPR4 and containing unique restriction enzyme sites

Thus the total length of the expected product is 619 aa and the calculated MW is 68.9 kDa. The C-terminal fragment cleaved at the trypsin cleavage site has a calculated MW of 64 kDa (corresponding to the 63 kDa C-terminal fragment of native PA with a 5 aa C-terminal addition).

As there were no appropriate restriction enzyme sites in pPA26 which could be used to construct this clone, PCR primers were designed to accomplish this. The strategy is illustrated in Figure 5.5. The primers ANTHPR3 and ANTHPR4 were designed to generate a PCR product for blunt-ended cloning into *Eco*RV cut pBluescript II KS+. The primers contained the appropriate nucleotides to reconstitute the *Eco*RV sites at each end of the insert. The primers were used in a standard PCR reaction using CsCl purified pPA26 as the template. Four 100 μl reaction tubes were run and the reaction mixes combined and run on a 0.7% agarose gel and the band of interest was purified using DEAE cellulose paper. The PCR product was blunted with Klenow and ligated into *Eco*RV cut pBluescript II KS+. The ligated DNA was electroporated into *E. coli* JM 109 and ampicillin resistant clones identified on L-agar plates supplemented with ampicillin. Colonies were picked, grown overnight in L-broth supplemented with ampicillin and the plasmid isolated. Two clones with the expected restriction pattern were identified except that they only contained one *Eco*RV site at the 5' end of the insert. These two clones were designated pVET4m and pVET6m and were shown to express a protein after IPTG induction which reacted with rabbit anti-PA antibodies by Western blotting.

ssDNA preps were made from both clones and the ssDNA was sequenced with the Universal primer (M13 -20) to establish the sequence at the 3' end of the insert. Both clones had lost the 3'T from primer ANTHPR4 to give GAATC rather than reconstituting the *Eco*RV site GATATC. As this did not alter the coding sequence, being 3' to the stop codon,

Figure 5.5 Strategy for the construction of pVET4m

A. PCR primers ANTHPR3 and ANTHPR4 were designed to amplify the 63 kDa C-terminal fragment of the PA gene from the Trypsin cleavage site to the stop codon. They contained non-homologous tails which would become inserted into the PCR product and introduce unique restriction sites at either end.

B. The PCR product was blunted with Klenow and cloned into *Eco*RV cut pBluescript II KS+. Although the primers were designed to recreate the two *Eco*RV sites at either end of the insert, the downstream site was not recreated due to a base deletion at this end.

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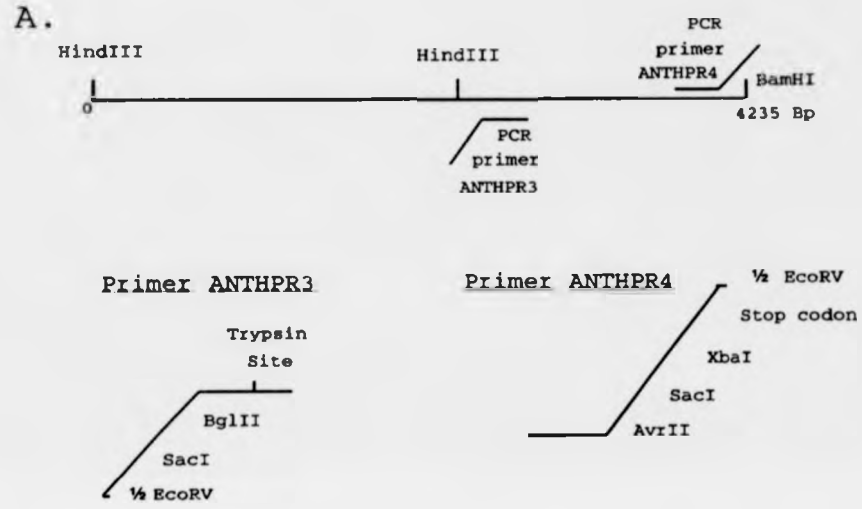

Figure 5.5 Strategy for construction of pVET4m

it was not considered significant. As both clones contained this difference it is possible that the PCR primer was synthesised without this nucleotide. To check for fidelity errors during PCR, the entire insert sequence for one of the clones, pVET4m, was verified by sequencing along the ssDNA template using sequencing primers PAS1-8 and dITPs and Taq chases where necessary to resolve CG compressions and premature termination due to template secondary structure.

The sequence obtained was compared to the reported sequence (Welkos *et al.*, 1988). Using the numbering used for the entire 4235 insert DNA in pPA26, a single base change was found at position 2743 (G to C) which would change a GAA codon to a CAA codon (glutamic acid to glutamine). This change was also found in pVET6m when this same region was sequenced. In order to establish if this was a PCR fidelity error, this same region was sequenced in pPA26, and DNA from the *B. anthracis* strains, New Hampshire, Sterne, Ames and Vollum (+1). The method used was to PCR amplify the 1.7 kbp region from all the templates and then dsDNA sequence this region in both directions using primers PAS7 and PAS7R. Primers ANPR3S and ANPR4S (see Section 3.2.2) were used in 3x100 μl PCR reactions which were cycled 30 times and then another aliquot of Taq enzyme added and the reactions cycled an additional 20 times. The products were gel purified and recovered using DEAE cellulose paper, alkali denatured and sequenced using standard methods with a Taq chase. This showed that the base change from the published sequence occurred with all templates. It was concluded that the reported sequence was incorrect.

5.3.1 Expression of PA from pVET4m

Whole cell lysates from *E. coli* JM 101 containing pVET4m (made using the methodology described in Section 4.3.2. for pORF1 clones) were subjected to SDS-PAGE and Western blotted. This is shown in Lane 3 of Figure 5.6.

As can be seen in Figure 5.6 an immunoreactive protein of the expected MW was expressed but also present were bands of lower molecular weights which presumably correspond to breakdown products of the protein. The difference between this recombinant protein and cleaved native PA is only 4.9 kDa and even on gels run with trypsin-cleaved native PA and recombinant PA a difference could not be distinguished. Thus it is not clear if the band seen represents the full-length recombinant protein or the cleaved C-terminal portion. The Western blot in Figure 5.6 was developed with mouse monoclonal antibodies

Figure 5.6 Western blot of expression of pVET4m and pVET4n in *E. coli* and *S. typhimurium*

Whole cell lysates, adjusted for cell number, were prepared from cultures of *E. coli* / pBluescript, pORF1, pVET4m and pVET4n. The cultures were induced with IPTG for 1 hour prior to harvesting the cells. *S. typhimurium* SL 3261 containing the same plasmids were grown similarly but without IPTG induction. The pellet from 10 ml of each culture was suspended in approximately 1 ml of 1xLB (proportional to the Abs_{600} of the culture) and boiled for 3 mins. This lysate was diluted 1:5 in 1xLB and 20 μl loaded in each well. This is equivalent to the protein from approx 1.5 x 10^8 cells loaded in each lane. Bio-Rad prestained MW markers were run in the middle lane and apparent MWs are shown. The gels were blotted onto PVDF membrane, blocked in Blotto, and incubated in a primary antibody of mouse anti-PA monoclonal antibodies, and a secondary HRP-conjugated anti-mouse IgG. The substrate used to visualised the bands was DAB.

Lane 1 *E. coli* JM 109 / pBluescript

Lane 2 *E. coli* JM 101 / pORF1

Lane 3 *E. coli* JM 101 / pVET4m

Lane 4 *E. coli* JM 109 / pVET4n

MW markers

Lane 5 *S. typhimurium* SL 3261 / pBluescript

Lane 6 *S. typhimurium* SL 3261 / pORF1

Lane 7 *S. typhimurium* SL 3261 / pVET4m

Lane 8 *S. typhimurium* SL 3261 / pVET4n

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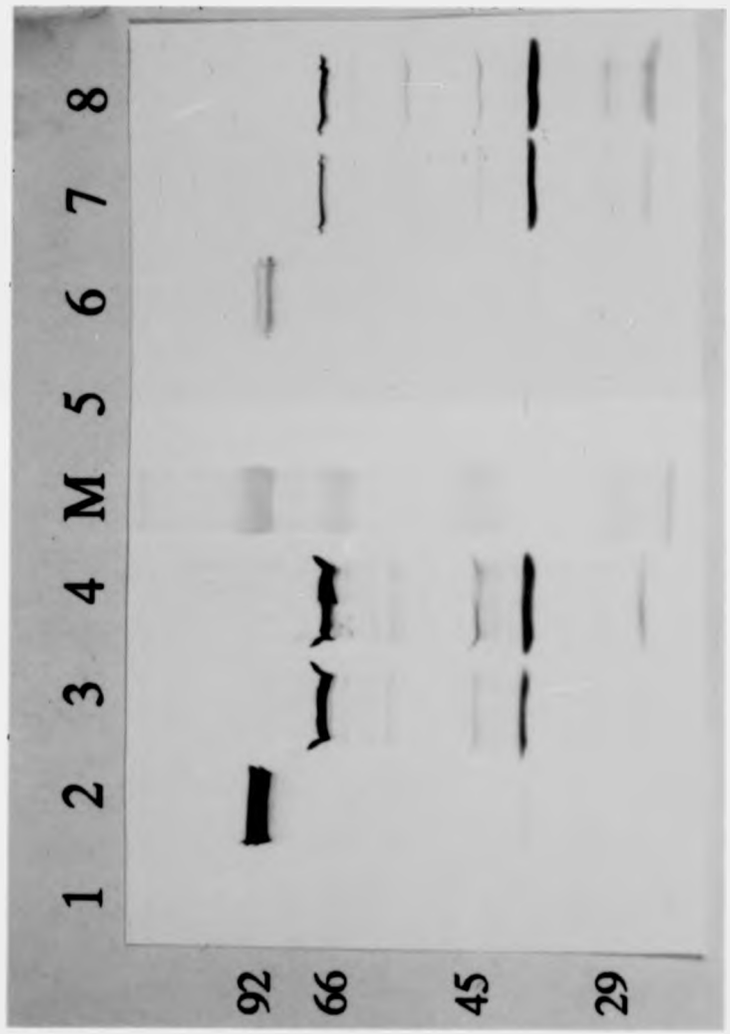

Figure 5.6 Western blot of expression of pVET4m and pVET4n in *E. coli* and *S. typhimurium*

which bind to the C-terminal end of the fragment. Therefore other breakdown product bands consisting of the N-terminal portion of the protein may not be visualised. Also this suggests that the 5 aa addition to the C-terminal end of PA expressed in pVET4m does not significantly alter the epitope that the mouse MAbs bind to.

The presence of so much of the immunoreactive protein within smaller MW bands suggests that it is being degraded. Fusions to β-galactosidase have been used to stabilise heterologous proteins against proteolytic degradation (Enfors, 1992). In this case only 34 amino acids of β-galactosidase remain and this may not be sufficient to stabilise the fusion. Thus it appears that fusion of this fragment of PA to β-galactosidase has not stabilised the construct although overall total expression levels may be higher than for pORF1. For comparison, expression from pORF1 is shown in Lane 2 of Figure 5.6. As the product from pORF1 appears to be all full-length, the bands are proportional to the level of expression. For pVET4m, the total expressed is the sum of the bands plus any protein bands which are undetected by the MAb.

When the phagemid pVET4m was electroporated into *S. typhimurium* SL 3261 a similar pattern of bands was seen, Lane 7, Figure 5.6. The overall level of expression appears to be lower in *S. typhimurium* than *E. coli*.

5.4 Construction of phagemid pVET4n

As pVET4m has an additional 5 aa coded in the 5' non-complimentary tail of ANTHPR4, this may have affected protein stability. A phagemid with a naturally configured C-terminal end to PA was constructed to compare expression, stability and immunogenicity with pVET4m. Plasmid pNIK1n (see Section 5.5) contains the coding region for the natural C-terminal end of PA and this can be removed from pNIK1n on a *Pst*I-*Pst*I fragment. This fragment can be cloned into *Pst*I cut pVET4m to replace the original *Pst*I fragment and so express PA with a natural C-terminal end. This is illustrated in Figure 5.7.

pVET4m was cut with *Pst*I, CIP treated and the vector and insert bands separated on an agarose gel. The vector band was recovered using Qiaex. pNIK1n was cut with *Pst*I and the insert gel-purified and recovered using Qiaex. Suitable ligation mixes were made and incubated overnight. The ligated DNA was electroporated into *E. coli* HB 101 (JM 101 or 109 should have been used as they have the *lacI*^{q} gene and so would have been able to regulate the promoter) and recombinant clones identified by plating on L-agar plates

Figure 5.7 Phagemid pVET4n

A. Phagemid pVET4n is similar to pVET4m except for the C-terminal end of the expressed PA fragment. In pVET4n the modified 5 amino acid addition is not present and the sequence is the same as for native PA. The fragment is expressed from the *lac* promoter in pBluescript as for pVET4m.

B. This shows the important restriction enzyme sites in pVET4n. It was constructed by replacing the *PstI-PstI* fragment with the same fragment from pNIK1n, a plasmid containing the native PA sequence at the C-terminal end.

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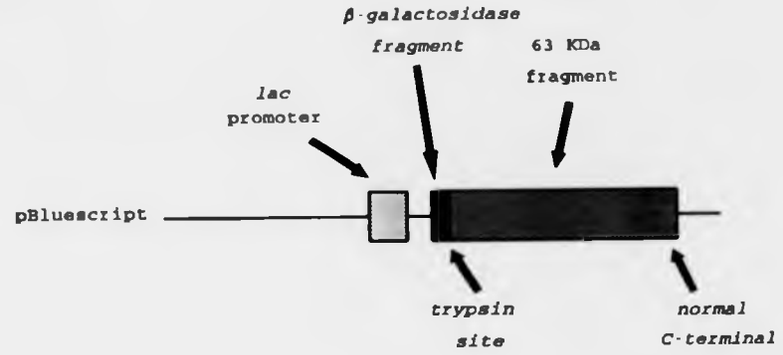
A.



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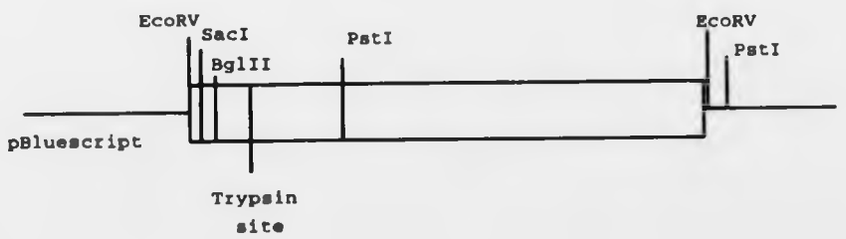

Figure 5.7 Phagemid pVET4n

supplemented with ampicillin. Clones with the insert present and in the correct orientation were identified using PCR. Primers PAS7R (originally a sequencing primer) and PA 1219 bind either side of the internal *Pst*I site and amplify a product of 400 bp if the clone has the insert in the correct orientation. 5 out of 18 clones were identified as positive by PCR and shown to produce an immunoreactive product of the expected MW on Western blotting with mouse anti-PA monoclonal antibodies. One of these clones was designated pVET4n ('n' for native PA C-terminal end) and shown to have the expected restriction enzyme profile.

5.4.1 Expression of PA from pVET4n

Expression of PA from pVET4n is shown in Figure 5.6. Lane 4 shows expression in *E. coli* and Lane 8 from *S. typhimurium* SL 3261. Although the bands may be slightly denser than for pVET4m, the overall pattern is the same. It seems that the modified C-terminal end was not responsible for the degradation of this fusion protein.

5.5 Construction of plasmid pNIK1n

In order to see if an alternative fusion system gives similar results, it was decided to express the same 63 kDa fragment of PA as a fusion protein from another vector. As discussed in Section 4.5, the *lac* promoter is not optimised for transcription. Therefore an alternative more active promoter was looked for and, after studying several commercially available plasmids, pTrc99A was chosen for further work. This contains the strong IPTG inducible promoter *trc* in a vector with the pBR322 *ori*. The *trc* promoter is similar to the *tac* promoter but with 17 bp spacing separating the -35 and -10 regions of the promoter, this leads to only 90% of the activity of the *tac* promoter (Brosius *et al.*, 1985). The vector does not express a particular protein, but the PA-63 would be preceded by 24 aa coded for by the linker sequences between the start codon and the beginning of the PA-63 ORF. One drawback with this plasmid in this project is that the *lacI*^{q} gene is present on the plasmid. This ensures good repression of the *trc* promoter, in case the protein is toxic, yet still allows *in vitro* expression by induction with IPTG. But the plasmid borne *lacI*^{q} would prevent constitutive expression from this promoter *in vivo*. Following an examination of restriction sites in the plasmid, it was found that the *lacI*^{q} gene contained the only two *Pvu*II sites in the constructs that were to be made. Thus removal of this *Pvu*II-*Pvu*II fragment offered a

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method to inactivate the repressor. If the constructs were found to be useful, this deletion would allow constitutive expression *in vivo*.

It was decided to clone the 63 kDa fragment of native PA (without the 5 aa C-terminal modification) downstream of this promoter so as to be able to compare expression of this fragment from both the *lac* promoter in pVET4n and the *trc* promoter. Construction of the plasmid is illustrated in Figure 5.8.

A phagemid, designated pBPA9, had been constructed to contain the 63 kDa fragment of PA, with the natural C-terminal end, in pBluescript II KS+. It was constructed using primers BPA3 and BPA4 to amplify the region required from pPA26 and, following Klenow blunting, it was cloned into *Eco*RV cut pBluescript. The ligated DNA was electroporated into *E. coli* JM 109 and recombinant clones selected for on L-agar plates supplemented with ampicillin. Clones with the insert were identified by PCR using the original primers BPA3 and BPA4, positive clones were then mini-prepped and restriction mapped using *Eco*RV and *Sac*I to identify clones with the insert in the correct orientation. A helper phage induced ssDNA preparation was made and the insert sequenced to confirm that no PCR induced mutations had occurred using sequencing primers PAS1-9, the universal primer (M13 -20) and the SK primer.

The *Kpn*I - *Xba*I fragment of pBPA9 contains an 18 amino acid linker coupled to the 63 kDa fragment of PA beginning with 3 aa then the trypsin cleavage site and the 568 PA ORF. This fragment was cloned into *Kpn*I-*Xba*I cut pTrc99A which adds another 6 amino acids expressed by the coding region of the polylinker before the 63 kDa fragment of PA. The final product would therefore be expected to have the 63 kDa fragment of PA as found in pVET4n, expressed as a fusion protein to a 24 aa linker protein.

pBPA9 and pTrc99A were cut with *Kpn*I and *Xba*I, separated on agarose gels and the appropriate bands recovered using Qiaex. The vector and insert were mixed in appropriate ratios, ligated, and electroporated into *E. coli* HB 101 (pTrc99A contains *lacI*^{q} on the plasmid). Colonies expressing PA were identified using colony blotting with IPTG induction as shown in Figure 5.9. A clone was designated pNIK1n and was shown to express a PA-immunoreactive protein and have the expected restriction enzyme profile.

Figure 5.8 Plasmid pNIK1n

A. The 63 kDa fragment of PA was expressed in vector pTrc99A. This contains the *trc* promoter in a pBR322 based vector. The construct pNIK1n expresses the 63 kDa fragment of PA after a 24 amino acid linker protein. The trypsin cleavage site is present and the construct has the native sequence at the C-terminal end.

B. The construct was made by cloning the *Kpn*I-*Xba*I fragment of plasmid pBPA9 into similarly cut pTrc99A. There is one internal *Pst*I site and two *Pst*I sites beyond the ORF for PA. One of these was from the polylinker of pBluescript and derives from pBPA9, and the other is in the pTrc99A polylinker.

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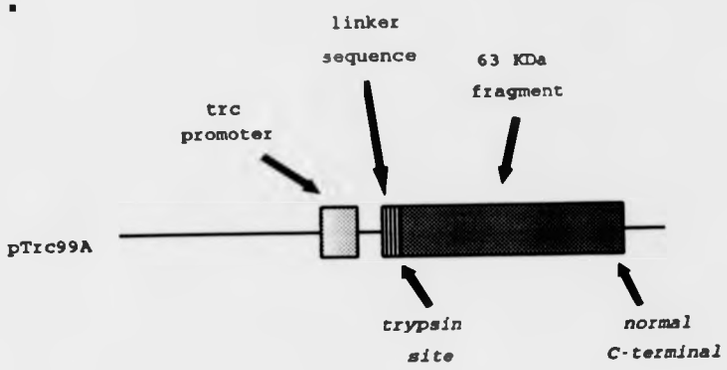
A.



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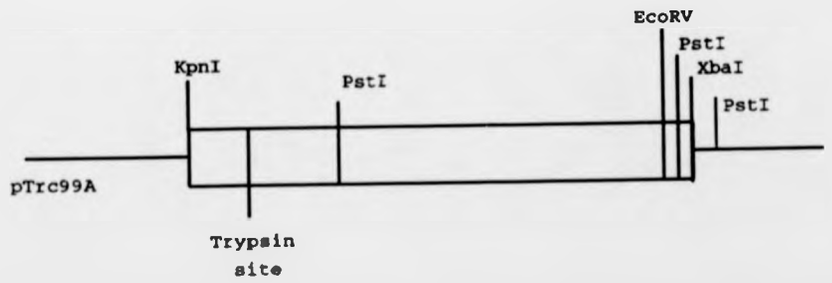

Figure 5.8 Plasmid pNIK1n

Figure 5.9 Colony blots to identify pNIK1n clones

During the construction of plasmid pNIK1n, an insert from pBPA9 was ligated into pTrc99A. Part of the ligation mix was electroporated into *E. coli* HB 101 and the bacteria plated onto ampicillin selection plates. Colonies that grew on these plates were streaked onto fresh plates and grown overnight to give 1 cm long ridges of bacteria. The bacteria were picked up on IPTG treated Hybond N membrane and incubated for 2 hours at 37°C. The bacteria were lysed with chloroform and treated with lysozyme and DNAse. The membranes were blocked with Blotto and incubated with a mouse anti-PA monoclonal. The MAb was detected with HRP linked anti-mouse IgG and the substrate DAB.

Four filters are shown. Positive colonies either appear as brown spots within the streak of bacteria, or as a larger brown area.

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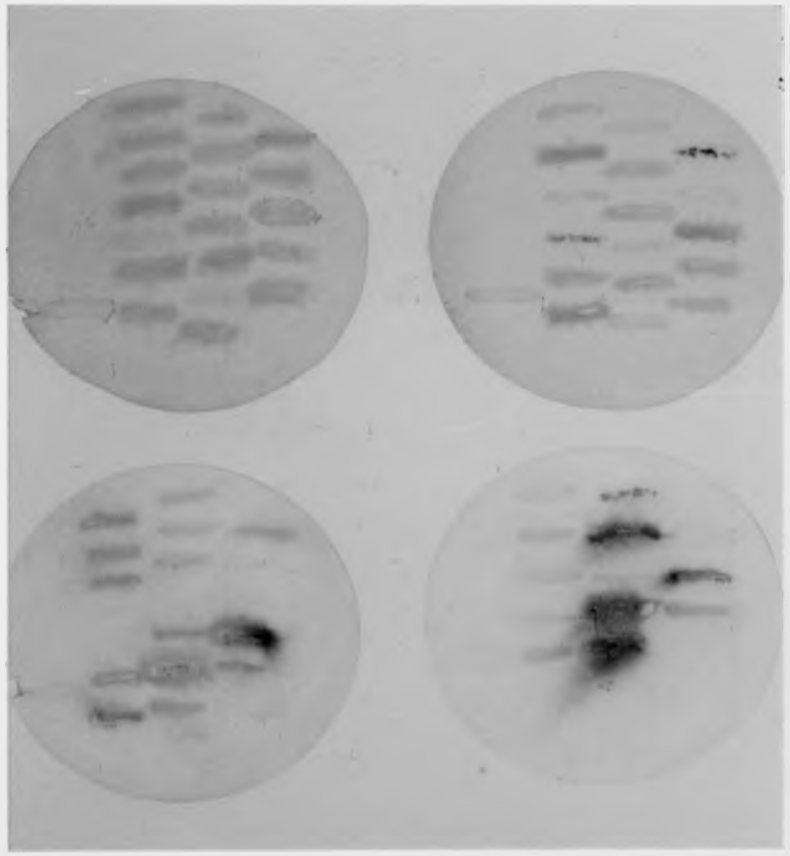

Figure 5.9 Colony blots to identify pNIK1n clones

5.5.1 Expression of PA from pNIK1n

Expression of PA from pNIKn was similar in *E. coli* and *S. typhimurium*, so only expression in *S. typhimurium* is shown. Expression was tightly regulated as the *lacI*^{q} gene is on the plasmid and so the repressor will be present at a high level. Without IPTG induction no immunogenic protein can be seen; Lane 6, Figure 5.10. With IPTG induction bands can be seen on the Western blot (Lane 8) which can be compared to expression from pORF1 and the pVET4 phagemids (Lanes 2, 3 and 4). The full-length product band is much less dense than for pVET4n suggesting that the protein is less stable and is degraded to give the lower MW protein visible on the blot. It was found that this plasmid was very unstable without antibiotic selection pressure; this is discussed further in Chapter 7.

5.6 Construction of plasmid pNIK1m

In order to complete the comparison of the effects of C-terminal changes identified between pVET4m and pVET4n, the same modified C-terminal end from pVET4m was cloned into pNIK1n. This construct is illustrated in Figure 5.11.

The *Pst*I fragment from pVET4m (containing the modified C-terminal) was gel purified and recovered using Qiaex. pNIK1n was cut with *Pst*I and CIP treated and then the vector band gel purified and recovered with Qiaex. The vector and insert were mixed in appropriate ratios and ligated overnight. The ligated DNA was electroporated into *E. coli* HB 101 (*lacI*^{q} on plasmid) and recombinant clones identified by plating on L-agar plates supplemented with ampicillin. Clones with the insert present and in the correct orientation were identified using PCR. Primers PAS7R (originally a sequencing primer) and PA 1219 bind either side of the internal *Pst*I site and amplify a product of 400 bp if the clone is correct. 4 out of 18 clones were identified as positive by PCR and shown to produce an immunoreactive product of the expected MW on Western blotting with mouse anti-PA monoclonal antibodies. One of these clones was designated pNIK1m and further characterised by restriction mapping.

5.6.1 Expression of PA from pNIK1m

Lanes 5 and 7 of Figure 5.10 show uninduced and induced cultures of *S. typhimurium* SL 3261 / pNIKm. This shows a similar pattern of bands to the pNIKn Western blot lanes with little full-length product but degradation product bands.

Figure 5.10 Western blot of expression of pVET and pNIK plasmids in *S. typhimurium* SL 3261

Whole cell lysates, adjusted for cell number, were prepared from uninduced cultures of *S. typhimurium* / pBluescript, pORF1, pVET4m, pVET4n, pNIK1m and pNIK1n. Separate cultures containing pNIK plasmids were also induced with IPTG for 1 hour prior to harvesting the cells. The pellet from 10 ml of each culture was suspended in approximately 1 ml of 1xLB (proportional to the Abs_{600} of the culture) and boiled for 3 mins. This lysate was diluted 1:5 in 1xLB and 20 μl loaded in each well. This is equivalent to the protein from approx 1.5 x 10^8 cells loaded in each lane. Bio-Rad prestained MW markers were run in the left lane and apparent MWs are shown. The gels were blotted onto PVDF membrane, blocked in Blotto, and incubated in a primary antibody of mouse anti-PA monoclonal antibodies, and a secondary HRP-conjugated anti-mouse IgG. The substrate used to visualised the bands was DAB.

MW markers
Lane 1 *S. typhimurium* SL 3261 / pBluescript
Lane 2 *S. typhimurium* SL 3261 / pORF1
Lane 3 *S. typhimurium* SL 3261 / pVET4m
Lane 4 *S. typhimurium* SL 3261 / pVET4n
Lane 5 *S. typhimurium* SL 3261 / pNIK1m uninduced
Lane 6 *S. typhimurium* SL 3261 / pNIK1n uninduced
Lane 7 *S. typhimurium* SL 3261 / pNIK1m induced
Lane 8 *S. typhimurium* SL 3261 / pNIK1n induced

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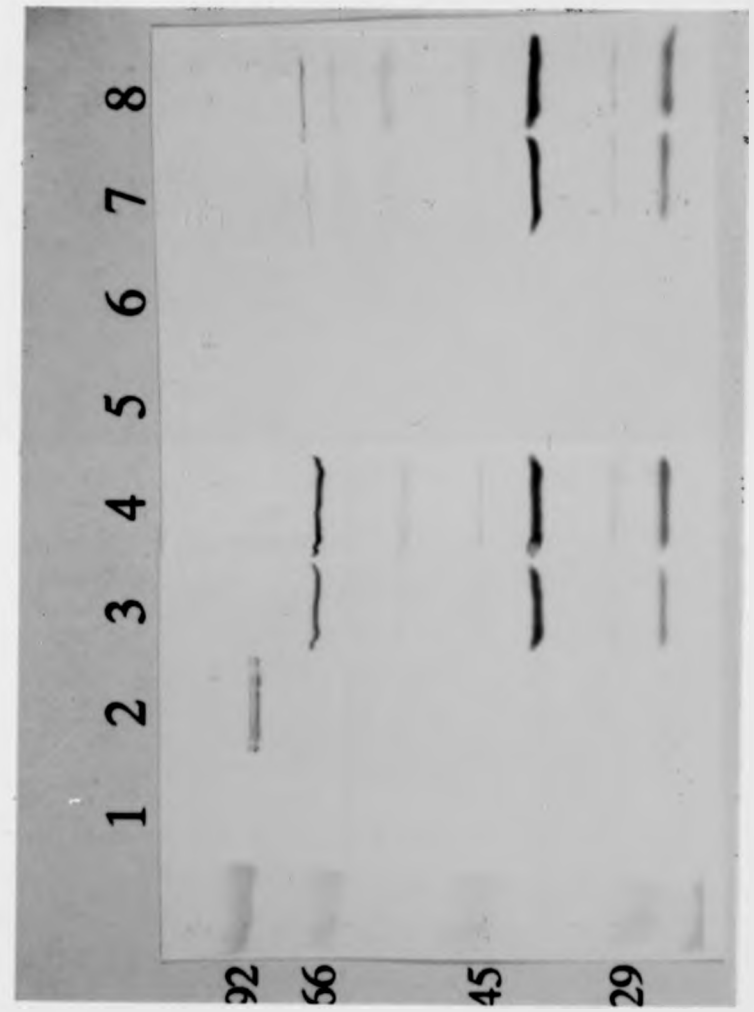

Figure 5.10 Western blot of expression of pVET and pNIK plasmids in *S. typhimurium* SL 3261

Figure 5.11 Plasmid pNIK1m

A. Plasmid pNIK1m is the same as plasmid pNIK1n except that it contains the *Pst*I-*Pst*I fragment of plasmid pVET4m which codes for the modified C-terminal end of PA.

B. This shows the restriction enzyme sites involved in the construction of plasmid pNik1m. The *Pst*I-*Pst*I fragment is from pVET4m.

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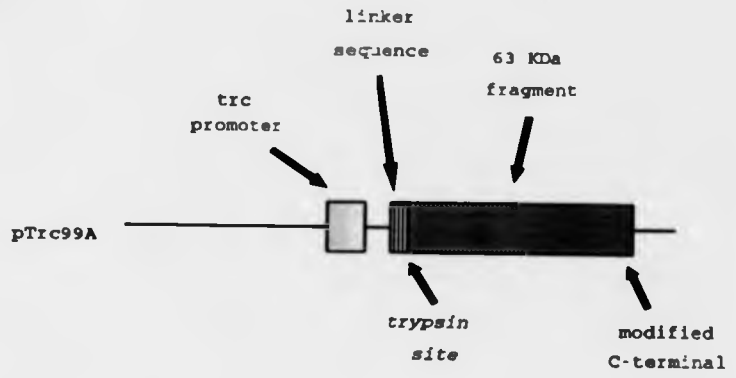
A.



B.

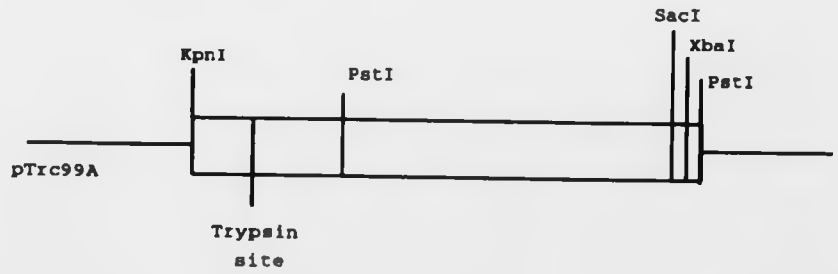


Figure 5.11 Plasmid pNIK1m

5.7 Conclusions

In Chapter 4 it was shown that altering the *B. anthracis* promoter to the *E. coli lac* promoter could significantly increase the level of expression of PA. As a further increase in expression was desirable the possibility of modifying the promoter and RBS was investigated but no obvious solution was apparent. In this Chapter the possibility that codon usage could be limiting translation was discussed. The PA gene's codon usage was found to be far from ideal for expression in *E. coli* or *S. typhimurium*. A strategy to chemically re-synthesise the gene was considered but found to be impractical in this study. It was therefore decided that the most practical strategy to try and increase expression was to fuse PA-63 to part of β-galactosidase. This strategy has produced mixed results. With phagemid pVET4m, the overall level of expression compared to pORF1 appears increased. This has been at the expense of producing a fusion protein that appears susceptible to degradation. As pVET4m had a modified C-terminal end it was possible that this could affect stability and activity. Recent evidence has shown the importance of the C-terminal end of PA in receptor interactions (Little & Lowe, 1991; Singh *et al.*, 1991) with deletions of as little as 12 aa completely abolishing PA binding to cells. These deleted proteins also do not bind monoclonals 3B6 and 14C7 which are known to bind to the cell-receptor site on PA. As pVET4m has an additional 5 amino acids coded in the 5' non-complimentary tail of ANTHPR4, this may affect the stability around this region. The 5 aa added to the C-terminal end (Tyr, Leu, Gly, Ala and Leu) are all hydrophobic. They were not selected for this attribute but are a consequence of the nucleotide sequence designated by the unique restriction sites added for further manipulations. The hydrophobicity of the C-terminal end of native PA is relatively neutral (as predicted by both Kyte-Doolittle and Hopp-Woods methods) but with this 5 aa modification both methods of prediction show increased hydrophobicity, see Figure 5.12. This might destabilise the conformation of the C-terminal end and affect the protein's degradation. Singh *et al.* (1991) showed that when they deleted 5-12 aa from the C-terminal end of PA-83 the proteins became very unstable. It is surprising that the monoclonal antibodies that are known to bind to the C-terminal region are still able to recognise this modified PA if the C-terminal end is so destabilised. In order to evaluate the effect of this modification a construct was made with a C-terminal end identical to the native PA. Phagemid pVET4n with a C-terminal end identical to PA had a similar pattern of degraded protein bands on Western blotting. It was concluded that the

Figure 5.12 Hydrophobicity plots for PA and modified PA

The hydrophobicity plots (Kyte-Doolittle and Hopp-Woods) for both native PA and modified PA with the 5 amino acid extension expressed by pVET4m were produced using the PROTEAN programme from DNASTAR for the Mac computer with the standard default settings. The same regions are shown for both proteins.

A. This shows both hydrophobicity plots for the 30 amino acids at the C-terminal of native PA.

B. This shows the hydrophobicity plots for the 35 amino acids at the C-terminal end of the modified PA expressed by pVET4m. The last five amino acids are all hydrophobic and so alter the hydrophobic moment of this region.

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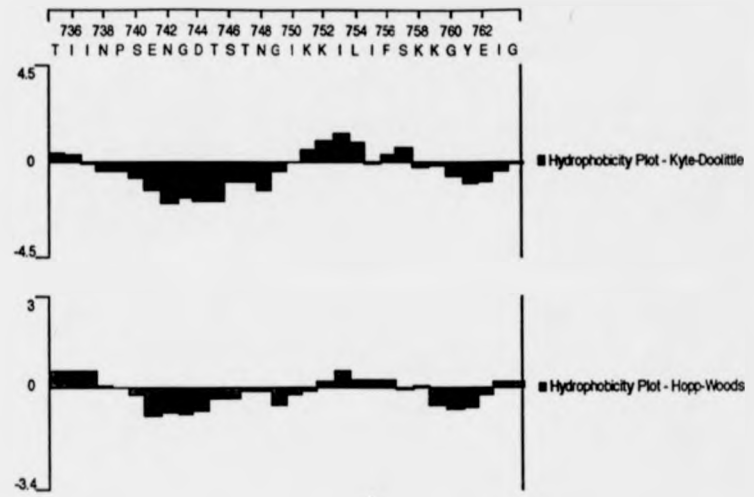
A. Native PA



B. Modified PA

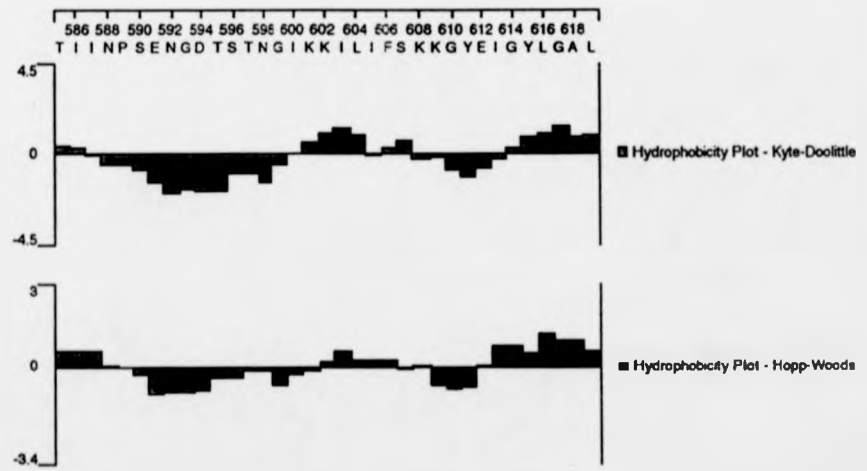


Figure 5.12 Hydrophobicity plots for PA and modified PA

modified C-terminal was not responsible for the protein's degradation.

Changing the nature of the peptide that PA-63 was fused to made the situation worse, with less full-length product from the pNIK plasmids compared to the pVET4 phagemids. The more active promoter *trc* did not obviously increase the expression level but direct comparisons to the pVET4 phagemids are not possible as the fusion protein was different.

Thus the strategy of expressing a fragment of PA has not produced the expected benefits. Although more protein was produced, it was less stable. It might be expected that the pORF1 product, with the PA gene signal sequence, would be secreted into the periplasm. Conversely, a β-galactosidase fusion protein would be expected to be cytoplasmically located. The different cellular location might effect the stability of the protein. There is a different proteolytic environment in the periplasm compared to the cytoplasm. Another alternative for the stability of full-length PA could be that the N-terminal 20 kDa fragment may have a role in stabilising the protein and protecting it from proteolytic degradation. As it would be useful to identify why full-length PA expressed by pORF1 is more stable, the cellular location of the PA will be addressed in the next chapter.

CHAPTER 6

Influence of cellular location on level of expression of PA

6.1 Introduction

The cellular location of expressed antigens can have a number of important consequences. These include the stability of the antigen, conformation of the antigen and immune responses to the antigen. Heterologous antigens are prone to proteolysis as they may be considered by the cell as aberrant proteins and so be degraded by bacterial proteases (such as Protease La, the product of the *lon* gene) designed for this purpose (Enfors, 1992). The protease environment in different cellular compartments varies and so the stability of a protein may differ with location (Oliver, 1987; Enfors, 1992). The conformation, and in some cases toxicity, of a protein can vary in different cellular compartments. The B-subunit of the heat-labile toxin (LT-B) of *E. coli* cannot form stable pentamers until it is exported and processed in the periplasm (Hirst *et al.*, 1992). Also the immune response to proteins in different cellular compartments may vary (Gao *et al.*, 1992), probably as a consequence of the manner of antigen presentation. It is therefore important to identify the location of the PA produced by the constructs and assess the possible effects of altering the cellular compartment of the heterologous protein.

Before the experiments to establish the location of PA could be carried out, the method of cell fractionation and verification of the fractions with enzyme assays was tested using a periplasmically located protein: the B-subunit of the heat-labile toxin (LT-B) of *E. coli.*

Gram positive protein signal sequences are known to function in Gram negative bacteria (Holt *et al.*, 1982; Sarvas, 1986; Curtiss, 1990). As phagemid pORF1 contains the *B. anthracis* PA gene signal sequence the PA protein might be exported into the periplasmic space. On the other hand, β-galactosidase is a cytoplasmic protein and the β-galactosidase fusion protein produced by pVET4m would be expected to be located cytoplasmically.

If the location of the above proteins were shown to be as expected, their location could be correlated with the stability of the proteins. One hypothesis that might explain the apparent stability of the PA produced from pORF1 was that a periplasmic location protected it from cytoplasmic proteolytic enzymes. This is in contrast to the 63 kDa product from pVET4m which appears to be degraded. Strategies that might control this proteolysis include expression in protease negative mutants, sequence modifications, fusions to proteins that encourage inclusion body formation or prevent proteolysis, or secretion (Enfors, 1992). If the stability of full-length PA is due to its secretion, the 63 kDa fragment might also be

protected if it is exported and it might then accumulate in higher amounts. This would improve the potential for this construct as a vaccine strain. The 63 kDa fragment might be exported if fused to a signal sequence such as the sequence for LT-B.

6.2 Cellular location of LT-B in *E. coli* HB 101

Naturally expressed *E. coli* LT-B is known to be located periplasmically (Witholt *et al.*, 1988). A plasmid containing the gene for LT-B (Schodel & Will, 1989) designated pFS2.2, contained the entire coding sequence for LT-B from a human isolate of *E. coli* (Clements *et al.*, 1986) placed downstream of the *lac* promoter, in pUC19. In order to show that the method for fractionating the cells was working as expected, it was first shown that this known periplasmic protein could be detected as expected.

From earlier work to identify PA production from pPA26, it was known that sonication could breakdown PA into fragments. A method of fractionation of cells which used three freeze/thaw cycles to disrupt spheroplasts was therefore chosen. It is usual to confirm the fractionation of the cells by assaying for both a cytoplasmic and a periplasmic enzyme. β-galactosidase can be used as the cytoplasmic marker enzyme and alkaline phosphatase as the periplasmic marker enzyme. As the gene for β-galactosidase has been deleted in some strains of *E. coli* to allow complementation, *E. coli* HB 101 was used for these studies. The appropriate plasmids were electroporated into *E. coli* HB 101. In order to induce large amounts of the enzyme alkaline phosphatase, the bacteria can be grown in modified M9 media which contains low phosphate and so induces expression of alkaline phosphatase. Unfortunately PA production could not be identified by Western blotting when this media was used. It was therefore found necessary to grow the bacteria in L-broth. This still led to expression of enough alkaline phosphatase to detect, although lower dilutions of the fractions were used in these assays than when *E. coli* was grown in modified M9 medium.

E. coli HB 101 containing pFS2.2 were grown overnight in L-broth supplemented with ampicillin. The culture was incubated for 2 hours with IPTG (1 mM) and then 40 ml of the culture was fractionated according to the described method, but with a 30 minute incubation with the TES/lysozyme solution. When alkaline phosphatase was assayed in the periplasmic and cytoplasmic fractions it was found that 90% was located in the periplasmic fraction. β-galactosidase assays showed that 97-99% of this enzyme was located in the

cytoplasmic fractions. The membrane fractions were optically dense and so difficult to use in enzyme assays. The location of LT-B within these fractions was assessed using a GM_1 ganglioside capture ELISA with an anti-LT-B MAb. This showed that approximately 10 times more LT-B was present in the cytoplasmic fraction than the periplasmic fraction. This unexpected result was probably due to an anomaly of this cell fractionation method combined with the ability of LT-B to aggregate at low ionic concentrations. It is known that LT-B is exported into the periplasm, proteolytically processed, folded, and only then assembled to form the pentameric structure that can bind with the GM_1 ganglioside (Witholt *et al.*, 1988; Hirst *et al.*, 1992). Thus the LT-B detected by the ELISA must have been exported correctly before it could produce a positive reaction in the ELISA, even if it was apparently found in the cytoplasmic fraction. It is known that in low ionic conditions periplasmically located LT-B can form large aggregates. These might be co-sedimented with the spheroplasts and so appear to be within the cytoplasmic fraction (pers. comm. Dr T Hirst). The use of only 30 mM Tris in the TES/lysozyme may mean that the LT-B was not solubilized. Therefore the experiment was repeated using 100 mM Tris in the TES/lysozyme. In this experiment 97% of the alkaline phosphatase activity was found to be located in the periplasmic fraction and 99% of the β-galactosidase activity was found in the cytoplasmic fraction. Using the high ionic concentration buffer the LT-B was now identified as being mostly (10^3 times more) in the periplasmic fractions.

It was concluded that the cell fractionation method was working satisfactorily, but the solubility of the heterologous protein could cause anomalous results if aggregates were formed. This potential problem would be considered when analysing the location of PA.

6.3 Cellular location of PA expressed by pORF1 in *E. coli* and *S. typhimurium*

The phagemid pORF1 expressed full-length PA with the *B. anthracis* PA gene signal sequence. It was shown to produce two closely spaced bands on Western blots (Figure 4.9) which possibly corresponded to the expected position of the gene product with and without a signal sequence. To evaluate the cellular location of PA from this construct, the phagemid was electroporated into *E. coli* HB 101. Cultures were grown overnight and the bacteria were fractionated using high ionic (100 mM) Tris TES/lysozyme. Enzyme assays showed 93% of alkaline phosphatase within the periplasmic fraction and 98% of β-galactosidase

within the cytoplasmic fraction. When the fractions were subjected to SDS-PAGE and Western blotted the PA-83 was present mainly in the periplasmic fraction (Figure 6.1, Lanes pORF-P versus pORF-C). On a Coomassie stained gel a band of the expected size was seen in the periplasmic fraction lane of pORF1 extracts (Figure 6.2).

Although it was not possible to confirm correct cell fractionation in *S. typhimurium* with these enzyme assays, a cell fractionation preparation of *S. typhimurium* SL 3261 containing pORF1 was made. Western blots and Coomassie stained gels are shown in Figure 6.3. Although the level of expression is apparently lower than in *E. coli* (a PA-83 band cannot be clearly identified on the Coomassie stained gel), the Western blot confirms that PA is exported in *S. typhimurium* and also shows a two banded appearance which supports this. In the periplasm the smaller of the two bands predominates whereas in the cytoplasm the larger of the two bands was stronger (lower photograph, Figure 6.3).

6.4 Cellular location of PA from pVET4m

In a similar experiment it was shown that the 63 kDa fragment expressed from pVET4m was cytoplasmically located (data not shown). This was expected for a β-galactosidase fusion protein. It had been shown (Figure 5.6) that pVET4m expressed a 63 kDa protein but also many smaller bands which represent degradation products. To investigate the effect of exporting the product of pVET4m, it was decided to try and export the 63 kDa PA fragment by fusing it to a signal sequence. If the protein being produced by pVET4m could be protected from degradation, then the amount accumulating might increase. This could improve the possibility of this construct inducing an immune response.

6.5 Construction of pSIG13

In order to evaluate the effect of exporting the PA-63, the gene coding for this region was fused to a signal sequence. Although it was shown that the *B. anthracis* PA gene signal sequence was active in *E. coli*, it was felt that an *E. coli* signal sequence might be more effective at exporting a heterologous protein. The signal sequence for LT-B was present in plasmid pFS2.2 which has been shown to export LT-B and to export LT-B fusion proteins into the periplasm of both *E. coli* and *S. typhimurium* (Schodel & Will, 1989). Plasmid pFS2.2 also had two *Sac*I sites with one located after the signal sequence and the other at the end of the ORF of LT-B (Figure 6.4). These allow the ORF to be removed and

Figure 6.1 Western blot to identify the cellular location of PA in *E. coli*

Plasmids pBluescript II KS+, pSIG13 and pORF1 were electroporated into *E. coli* HB 101. Clones containing the plasmids were identified. Cultures were grown overnight in 50 ml L-broth supplemented with ampicillin to Abs_{600} of approximately 4. 30 ml of each culture were fractionated as described in Chapter 2 using 1.5 ml of 100 mM Tris in the TES/lysozyme solution for 30 mins incubation. Each fraction was adjusted to 1.5 ml. The fractions were assayed for alkaline phosphatase and β-galactosidase activity to verify the effectiveness of the cell fractionation procedure. 50 μl of each fraction was mixed with 50 μl 2xLB and boiled. The pORF1 fractions were diluted 1:20. Bio-Rad prestained MW markers were loaded on the left of the gel and apparent MWs are shown (kDa). The samples were subjected to SDS-PAGE through an 8% gel. transferred onto PVDF membrane, blocked with Blotto, incubated with mouse anti-PA MAbs followed by HRP-conjugated anti-mouse IgG which was detected with DAB.

pBS	pBluescript II KS+
pSIG13	signal sequence and 63 kDa PA fragment
pORF	pORF1, *lac* promoter with PA signal sequence and full-length PA (1/20th loaded compared to pBS and pSIG13)
P	periplasmic fraction
C	cytoplasmic fraction
M	membrane fraction

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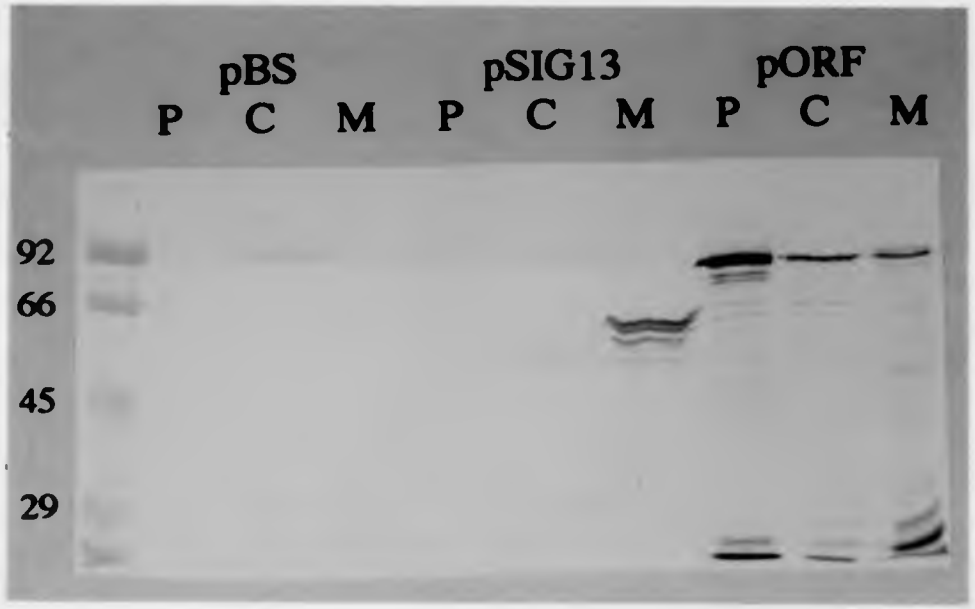

Figure 6.1 Western blot to identify the cellular location of PA in *E. coli*

Figure 6.2 Coomassie stained gel showing cellular location of PA in *E. coli*

E. coli HB 101 containing plasmids pBluescript II KS+, pSIG13 and pORF1 were grown overnight. Cell fractions were assayed for alkaline phosphatase and β-galactosidase activity to verify the effectiveness of the cell fractionation procedure. 50 μl of each fraction was mixed with 50 μl 2xLB and boiled. Bio-Rad prestained MW markers were loaded on the left of the gel and apparent MWs are shown (kDa). The samples were loaded in different amounts to prevent overloading, in the ratio of P:C:M of 10:1:2.5 with the pORF1 periplasmic fraction amount equivalent to 20 times the amount loaded on the respective Western blot lane (Figure 6.1). The samples were subjected to SDS-PAGE through an 8% gel and the gel was stained with Coomassie Blue.

pBS	pBluescript II KS+
pSIG13	signal sequence and 63 kDa PA fragment
pORF	pORF1, *lac* promoter with PA signal sequence and full-length PA

P	periplasmic fraction
C	cytoplasmic fraction
M	membrane fraction

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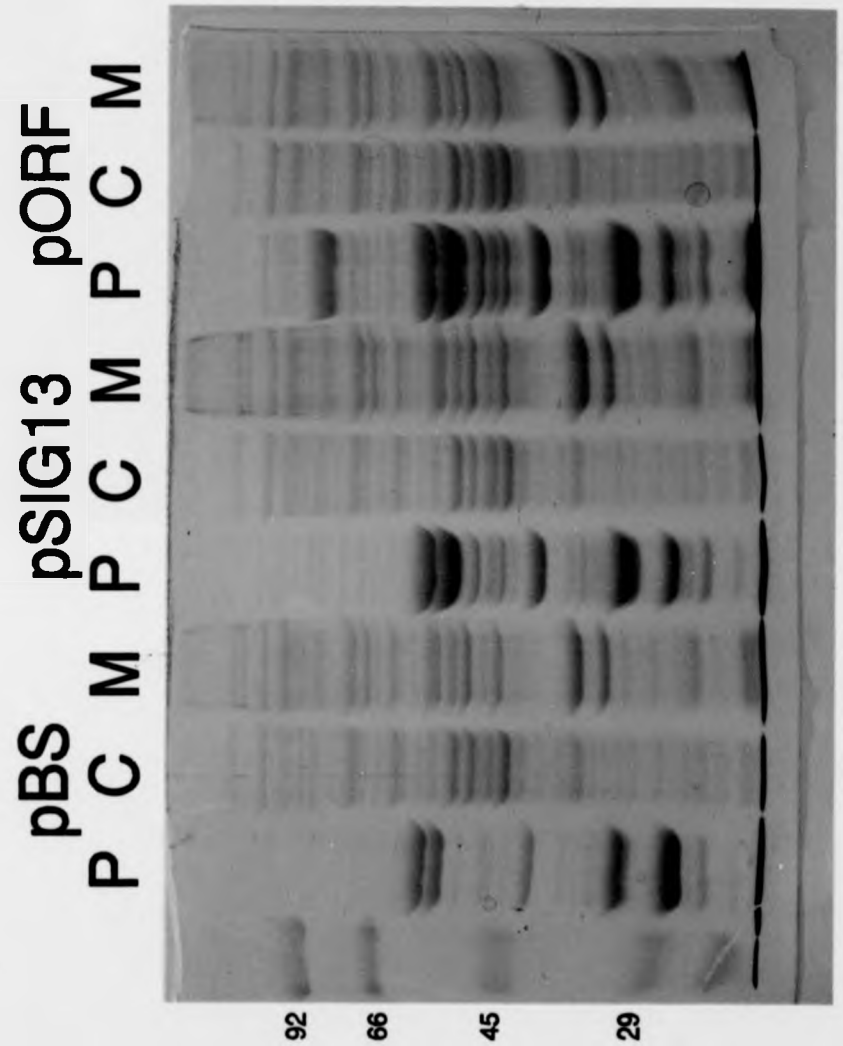

Figure 6.2 Coomassie stained gel showing cellular location of PA in *E. coli*

Figure 6.3 Western blot and Coomassie stained gel showing cellular location of pORF1 expressed PA in *S. typhimurium* SL 3261

The top photograph show a Western blot and matching stained gel of different cellular fractions of *S. typhimurium* SL 3261 / pORF1. The lower photograph shows an enlargement of the bands present in the Western blot around the level of the 92 kDa MW marker.

S. typhimurium SL 3261 / pORF1 was grown overnight, without shaking, in 50 ml L-broth supplemented with ampicillin to an Abs_{600} of 1.4. 30 ml of culture was pelleted and the cells fractionated as described in Chapter 2 using 1.5 ml of 100 mM Tris in the TES/lysozyme solution for 30 mins incubation. Each fraction was made to 1.5 ml.

For the Western blot 10 μl of each fraction was mixed with 90 μl 1xLB and boiled. Bio-Rad prestained MW markers were loaded on the left of the gel and apparent MWs are shown (kDa). 20 μl of all the 1:10 diluted samples were subjected to SDS-PAGE on an 8% gel, blotted onto PVDF membrane, blocked with Blotto, incubated with mouse anti-PA MAbs followed by HRP-conjugated anti-mouse IgG which was detected with DAB. The PA is mainly present in the periplasmic fraction, and in the enlargement it can be seen to have a lower MW than the PA band in the cytoplasm. This may represent the cleavage of the signal sequence.

For the Coomassie stained gel, Sigma 6H MW were run as standards. The samples were loaded in different amounts to prevent overloading. in the ratio of P:C:M of 10:1:5 with the cytoplasmic fraction amount equivalent to the amount loaded on the Western blot. The samples were separated by SDS-PAGE and the gel was stained with Coomassie. It is difficult to identify a band corresponding to the PA present.

P	periplasmic fraction
C	cytoplasmic fraction
M	membrane fraction

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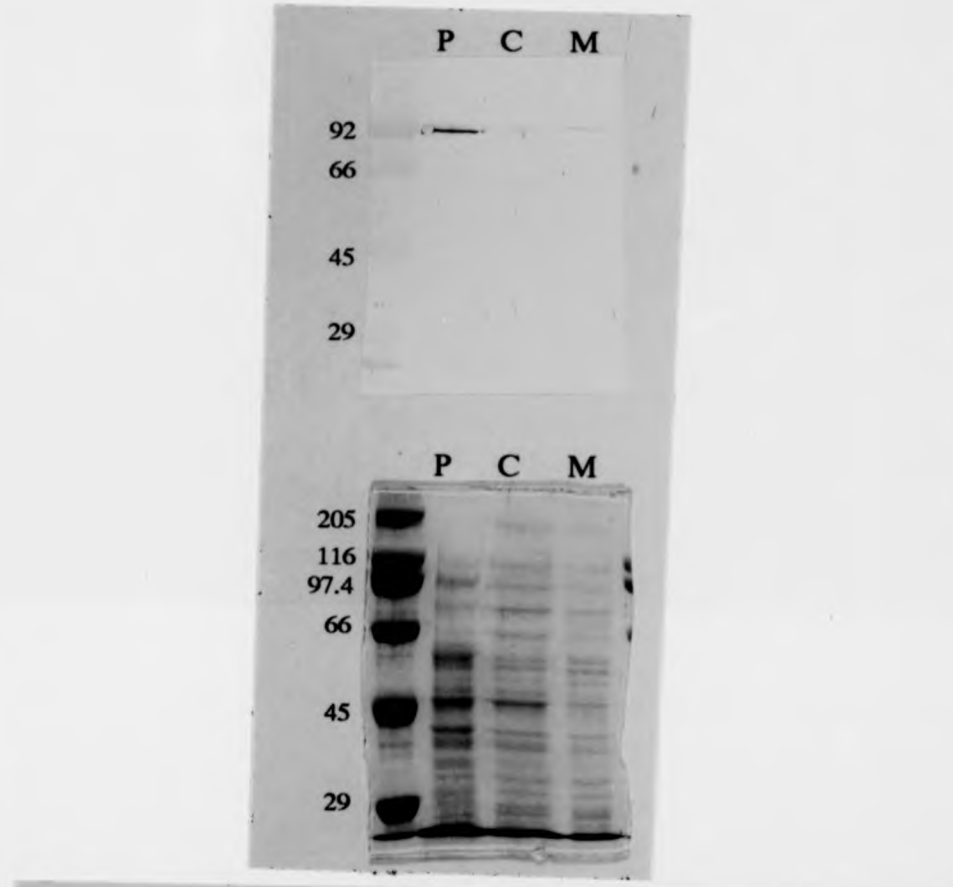

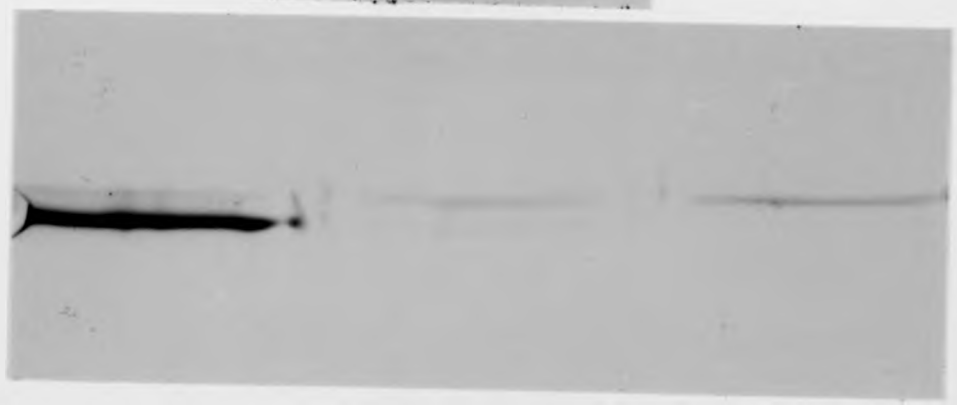

Figure 6.3 Western blot and Coomassie stained gel showing cellular location of pORF1 expressed PA in *S. typhimurium* SL 3261

Figure 6.4 Construction of pSIG13

A. Physical map of plasmid pFS2.2 (Schodel & Will, 1989), a pUC19 based plasmid containing the open reading frame for the *E. coli* heat-labile enterotoxin B subunit (LT-B) expressed from the *lac* promoter.

B. A blunt-ended fragment containing the 63 kDa coding region of PA was cut from the plasmid pVET4. This *Eco*RV-*Sma*I fragment contained a short region of β-gal and the trypsin cleavage site at the 5' end and the stop codon for the open reading frame at the 3' end. Plasmid pFS2.2 was cut with *Sac*I and gel purified. The *Sac*I overhangs were blunted with Klenow and dephosphorylated with CIP. The pVET4 fragment was ligated to the vector and electroporated into *E. coli* JM109. Colonies with the insert in the correct orientation were identified by PCR and restriction mapping. One clone with the correct restriction pattern was designated pSIG13.

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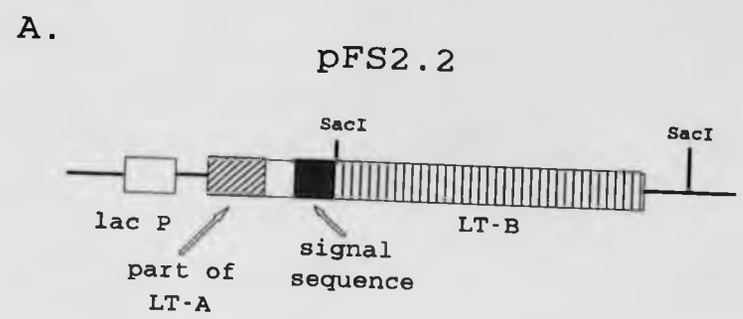

Figure 6.4 Construction of pSIG13

replaced by another ORF. In order to keep the insert of pVET4m in frame with the signal sequence, a blunt ended *Eco*RV - *Sma*I fragment of pVET4m was isolated from an agarose gel and Qiaex purified. *Sac*I digested pFS2.2 was blunted with Klenow and CIP treated to prevent recircularization of the vector. The vector and insert were ligated and electroporated into *E. coli* JM 109. Clones with the insert in the correct orientation were identified by PCR and restriction mapping. A clone expressing PA of about 63 kDa size was identified and the plasmid designated pSIG13. The sequence at the vector-insert junction was verified by sequencing. The plasmid was electroporated into *S. typhimurium* LB 5010, recovered and electroporated into *S. typhimurium* SL 3261. Whole cell lysates were made and PA expression compared to pVET4m. This is shown in Figure 6.5, lanes 3 and 4. The calculated MW of the pSIG13 product was 67.6 kDa if the signal sequence was attached and 65.2 kDa if the signal sequence was cleaved. From the Western blot it appears that the pSIG13 product is slightly smaller than the predicted MW of the product from pVET4m (68.9 kDa) as expected; but whether the signal sequence is present cannot be resolved.

6.6 Cellular location of PA from pSIG13 in *E. coli*

The cellular location of PA expressed by pSIG13 is shown in Figure 6.1 and Figure 6.2. Most of the PA was located with the membrane fraction. This may be because the protein is complexed with the membrane or because it was insoluble and so co-sedimented with the membrane fraction. The signal sequence did not appear to cause the protein to be exported into the periplasmic space.

6.7 Conclusions

The full-length PA expressed by pORF1 was exported into the periplasm and it was concluded that the *B. anthracis* PA gene signal sequence is functional in *E. coli*. This was supported by the size difference seen between the PA in the cytoplasm and the periplasm. The signal sequence was uncleaved in the small amount of cytoplasmically located PA but the periplasmic PA was a smaller protein band and presumably lacked a signal sequence. The observation that most of the PA has accumulated in the periplasm, in the signal peptide cleaved form, suggests that the protein is being rapidly and successfully secreted.

The 63 kDa fragment of PA expressed cytoplasmically as a β-galactosidase fusion from pVET4 is less stable as smaller MW products are visible on a Western blot. This

Figure 6.5 Western blot showing expression of PA from pSIG13 in *S. typhimurium*

S. typhimurium SL 3261 alone or containing either pPA26, pVET4m or pSIG13 were cultured overnight in 5 ml L-broth supplemented with ampicillin if appropriate. The Abs_{600} readings of the cultures were used to adjust the amount pelleted so that equal cell numbers could be lysed. The pellets were resuspended in 100 μl of 1xLB and boiled. 10 μl of the lysates were loaded onto a 8% gel with Bio-Rad prestained MW markers and subjected to SDS-PAGE, blotted onto PVDF membrane, blocked with Blotto, incubated with mouse anti-PA MAbs followed by HRP-conjugated anti-mouse IgG which was detected with DAB.

Bio-Rad MW markers (apparent MWs shown in kDa)
Lane 1 *S. typhimurium* SL 3261
Lane 2 *S. typhimurium* SL 3261 / pPA26
Lane 3 *S. typhimurium* SL 3261 / pVET4m
Lane 4 *S. typhimurium* SL 3261 / pSIG13

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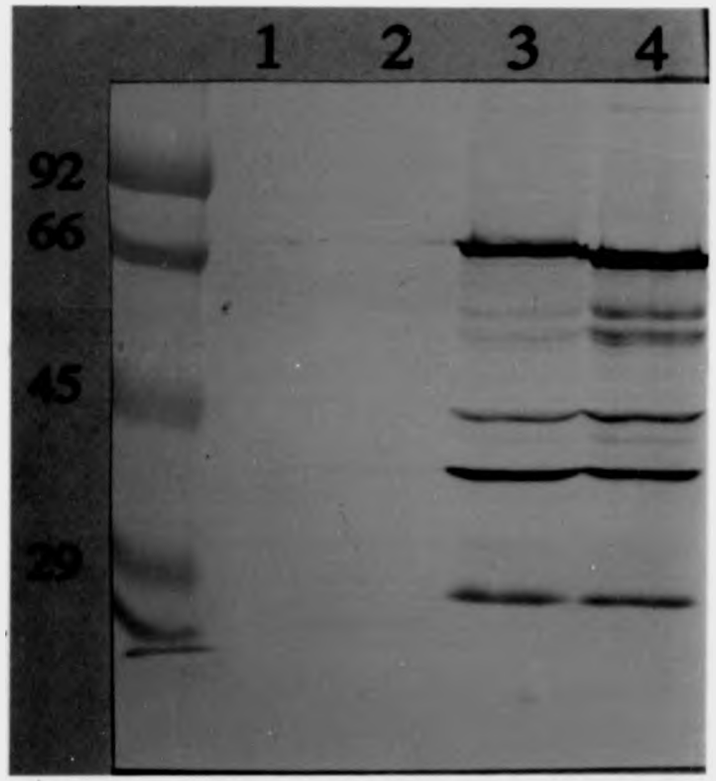

Figure 6.5 Western blot showing expression of PA from pSIG13 in *S. typhimurium*

degradation of the expressed product could prevent the accumulation of large amounts of heterologous protein and would also affect the immunogenicity of the protein in as far as it reduces the possibility of conformational epitopes being presented to the immune system. This degradation is not apparent in the PA expressed from pORF1 (see Figure 5.6). This could be due to it being exported into the periplasm where it would be subjected to a different protease environment than in the cytoplasm, or due to a stabilising effect of the 20 kDa fragment. The amount of immunoreactive protein present in the degradation product bands may account for more protein than is found in the 63 kDa band. It was hypothesised that exporting the 63 kDa fragment into the periplasm might reduce degradation. The strategy of fusing it to an *E. coli* signal sequence failed to lead to export of this heterologous antigen. In order to discuss this failure of export it is necessary to consider the mechanism of protein export in more detail.

Proteins are targeted for export by an N-terminal signal sequence. A comparison of signal sequences reveals a surprising lack of strict homology, except within the cleavage site, but signal sequences do show common areas of residue type (Gierasch, 1989). The N-terminal region carries a net positive charge and is followed by a hydrophobic core region. The cleavage site is usually 5-7 residues. The lack of specific patterns suggests that binding between the signal sequence and other proteins or membranes occurs in an unspecific way. Indeed, even random hydrophobic regions can act as a signal sequence (Schatz & Beckwith, 1990). On the other hand, it is known that point mutations can lead to loss of function, usually when a charged residue is introduced within the hydrophobic core (Gierasch, 1989).

Many genes associated with protein export have been characterised. This has led to a proposed model for export. The signal sequence targets the protein for export, but the protein must not be configured or folded in such a way that its translocation through the membrane is inhibited. Once through the membrane the cleavage site must be recognised by a signal peptidase so that the mature protein can be released (Gierasch, 1989). The *secB* protein is thought to bind to the signal sequence of some protein chains as they form and have a 'chaperone' role in preventing early folding of the protein. Other proteins may also have an anti-folding role, such as Trigger Factor and the GroEL heat shock protein (Saier *et al.*, 1989). The precursor-SecB complex then binds to SecA which recognises both regions of SecB and the signal sequence (Bassford *et al.*, 1991). The SecA binds to the membrane complex of SecE/SecY and translocation occurs driven by ATP hydrolysis and

the proton motive force (Bassford *et al.*, 1991). SecD, SecF and the signal peptidases play a role in later steps of the process (Schatz & Beckwith, 1990).

As many cytoplasmic proteins can be exported by attaching them to a signal sequence, it seems that the mature protein does not contain any essential regions for export. However, features of the mature protein can inhibit export. The amino acid sequence at the N-terminal end of the mature protein can influence export. A net positive charge in the first 5 amino acids of the mature protein can severely reduce export (Schatz & Beckwith, 1990) and it has been suggested that positively charged segments adjacent to signal sequences are preferentially retained on the cytoplasmic side of the membrane (Saier *et al.*, 1989). Similarly, alterations to the amino acids at the C-terminal end of LT-B were found to delay or prevent its release from the membrane export mechanism and this may be due to changes in the hydrophobicity of the molecule (Hirst *et al.*, 1992). Jagusztyn-Krynicka *et al.* (1993) showed that addition of a five amino acid C-terminal linker to *E. coli* LT-B , containing four hydrophobic amino acids, specified a protein that was not exported into the periplasm and was cytotoxic when induced.

With some proteins the inhibition of export is attributed to the protein adopting a secondary structure which is not compatible with translocation. The best-studied example of this is β-galactosidase which enters the translocation mechanism but becomes embedded in the cell membrane and, at high levels, blocks the cell export mechanism (Schatz & Beckwith, 1990). The success of the translocation step depends on the export-competent conformation of the protein. This is supported by the chaperone role of SecB and other similar proteins which are thought to prevent the formation of secondary structure in the protein which could prevent translocation. Also the close coupling of translation and export may be designed to decrease the potential for undesirable protein folding to occur.

Therefore protein export may be inhibited by an inappropriate charge gradient, or by the protein adopting a secondary structure which is incompatible with translocation (Saier *et al.*, 1989).

The signal sequences for PA and LT-B are shown in Figure 6.6. The signal sequence for PA is 29 amino acids long. The average length of the signal sequence in *Bacillus* species is longer, usually between 30-40 amino acids. This is in comparison to an average length of 22 amino acids in other species (Sarvas, 1986; Watson, 1984). The PA signal peptide also has an N-terminal region which is rich in basic residues as has been

Figure 6.6 Signal sequences of PA and LT-B

Amino acid sequence of the signal sequences of *E. coli* LT-B and PA. Above each sequence the N-terminal positively charged amino acids are marked with '+' and the predicted cleavage site with 'V'. Below the sequence, hydrophobic amino acids in the signal sequence are marked with 'H'.

The *E. coli* signal sequence is from Yamamoto & Yokota (1983).

The PA signal sequence is from Welkos *et al.* (1988).

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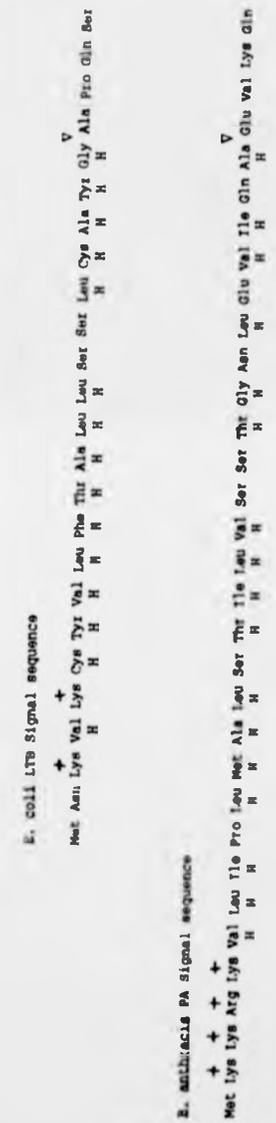

Figure 6.6 Signal sequences of PA and LT-B

reported for other *Bacillus* signal peptides (Sarvas, 1986). The cleavage site also conforms to the general pattern for prokaryotes. Cleavage occurs after an alanine residue as found in other *Bacillus* cleavage sites (Sarvas, 1986). Many Gram positive signal sequences are known to function satisfactorily in *E. coli* and from an analysis of the signal sequence of PA it would appear to meet the criteria of a Gram negative signal sequence. Therefore it would be expected to target PA for export in *E. coli*. The fact that the full-length PA from pORF1 is exported suggests that the PA is not only targeted for export but also is conformed in an export-competent structure.

The *E. coli* LT-B signal sequence is 21 amino acids long and conforms to the characteristic pattern for signal sequences of *E. coli* (Von Heijne, 1983; Watson, 1984; Oliver, 1987). The failure of the sequence to effect the export of the 63 kDa fragment of PA is therefore unlikely to be due to the sequence not targeting the protein for export. When the signal sequence-PA-63 fusion was made the amino acids downstream from the cleavage site were modified. An analysis of the site in pSIG13 shows that it still conforms to the pattern of amino acids at cleavage sites (Von Heijne, 1983). Thus a failure of cleavage, which could produce a failure of export and the appearance of the heterologous protein with the membrane fraction, seems unlikely. It appears that it is more likely to be the features of the mature protein, causing an inhibition of translocation, which is responsible for the failure of export.

It has been shown that export can be inhibited by a net positive charge close to (within about 5 amino acids) the N-terminal end of the mature protein (Schatz & Beckwith, 1990). In the fusion protein produced by pSIG13, the positively charged trypsin cleavage site RKKR is located 9 amino acids from the cleavage site. This may be too far from the cleavage site to affect export adversely but the presence of four positively charged amino acids may be enough to alter the charge profile in the region. This may cause this region to be retained on the cytoplasmic side of the membrane (Saier *et al.*, 1989).

As reported, changes to the C-terminus of LT-B also adversely affected export (Hirst *et al.*, 1992; Jagusztyn-Krynicka, 1993). Another explanation for the failure of export of PA expressed by pSIG13 is that the alterations to the PA molecule made it more hydrophobic and increased its potential for remaining bound to the membrane. As discussed in Section 5.7 the modifications to the C-terminal end of PA to introduce restriction enzyme sites created a more hydrophobic C-terminal end to PA expressed by pVET4m. This same

sequence occurs in pSIG13. It is possible that this alteration may interfere with export of the PA from pSIG13. Although these features may affect export, precise modifications to both regions would have to be made to determine this. It is also possible that the failure of export is due to the structure adopted by the fragment of PA being incompatible with export.

In conclusion, although many proteins have been successfully exported by the addition of a signal sequence (Smith *et al.*, 1987; Gierasch, 1989; Gao *et al.*, 1992), other workers have also found difficulties with export of proteins by this method (Strahan, 1992; Su *et al.*, 1992). The finding that PA from pSIG13 is detected mostly in the membrane fraction may suggest that it is fused with the membrane and so may have been targeted for export but failed to pass through the membrane and become stuck in the secretory mechanism. The signal sequence targets the protein chain for export but the configuration and charge distribution of the protein are important if it is to pass through the membrane.

Due to the failure to export 63 kDa PA into the periplasm, it is not possible to evaluate the effect of the periplasmic environment on this protein fragment. Approaches to effect the export of the fragment would include trying other signal sequences, fusions to the C-terminal end of an exported protein, or modifications to the C-terminal end of PA to affect its hydrophobicity. Alternatively, as it is known that the *B. anthracis* PA gene signal sequence can export the full-length PA protein, this might be the best starting place for a rational investigation of export of PA fragments.

As it is known that cytoplasmic heterologous antigens can generate immune responses, export is not essential for a candidate vaccine strain. The reason for attempting to export the 63 kDa fragment of PA was to try and prevent proteolytic cleavage. As time does not permit a thorough and rational evaluation of export, this approach will not be pursued.

CHAPTER 7

Selection of candidate recombinant vaccine organisms

7.1 Introduction

Various plasmids and phagemids have been constructed to express all, or part of PA in *S. typhimurium* SL 3261. For various reasons, none of these recombinant organisms are ideal for use as a vaccine. They all rely on antibiotic selection to maintain the plasmids, the level of expression is not very high and *S. typhimurium* SL 3261 only has a single attenuating lesion. However, before additional effort is put into solving these problems, it would be useful to show that PA expressed by *S. typhimurium* can induce protective immune responses. Even if only limited protection occurs with these preliminary recombinant organisms, further work to develop a vaccine strain would be justified.

Therefore the recombinant organisms must now be assessed to select the most suitable for animal studies of protection. The factors to be considered in the selection are the stability and level of PA expression of the plasmids *in vitro* (as an indicator of their *in vivo* stability and expression). Additionally, some preliminary animal data was available from an experiment using three of the plasmids. The potential stability of the plasmid *in vivo* can be assessed by culturing bacteria without antibiotic selection and then plating out the culture on plates, with and without antibiotic, so that the percentage of cells that are still antibiotic resistant can be evaluated. The level of heterologous antigen expression may be evaluated at different phases of the growth cycle. As expression may be detrimental to the bacteria and so slow or prevent growth, expression can be related to growth curves for cultures. From all the available data the most suitable construct for animal testing can be identified.

7.2 Stability *in vitro*

The stability of the plasmids *in vitro* was determined by picking a colony from an ampicillin selection plate and resuspending the colony in 5 ml of L-broth to form the stock culture. Part of this was stored as a -70°C glycerol stock and used in subsequent experiments. In order to assess the percentage of bacteria in the stock culture that were antibiotic resistant, 100 μl aliquots were plated onto L-agar or L-agar supplemented with ampicillin and the ratio of colonies on each plate determined.

100 μl of the stock culture was inoculated into media, with or without ampicillin/IPTG, and the cultures incubated overnight at 37°C. Different incubation conditions were used to give three different rates of growth. The cultures were either grown

in a static sealed Universal (in 10 ml of media), a shaken sealed Universal (10 ml) or a shaken flask (50 ml). These different conditions led to increasing cell densities by the end of the growth phases reflected by the final Abs_{600} readings in the approximate ranges of 0.3-0.5, 0.9-1.0 and 3-4, respectively. After incubation the cultures were diluted in ice-cold PBS to give an approximate concentration of 10^4 cells/ml. This dilution was plated out using a spiral plater onto L-agar plates or L-agar supplemented with ampicillin. The colony distribution was determined by a laser colony counter and the count computed by using BEN software. The ratio of the colony counts on the plates was used to calculate the percentage of colonies that were ampicillin resistant.

7.2.1 Stability *in vitro* Results

The results are shown in Table 7.1, A. (and discussed by vector group below).

pPA26 is pBR322 based and only expressed PA at low levels. It was relatively unstable in *S. typhimurium* SL 3261 when grown without antibiotic selection pressure with only 30% of bacteria retaining the plasmid after growth with aeration.

The pBluescript phagemids fall into two groups. The phagemid without an insert and the construct pCB1 which only expresses PA at low levels, were quite stable with 95 to 100% retaining the plasmid even after aerated growth in a flask. Constructs expressing higher levels of PA were less stable. pORF1 was the most unstable; only 1% of bacteria retained the phagemid even after a static incubation to low cell densities. In fact the starter culture made by picking a colony from an ampicillin selection plate was found to only contain 50% ampicillin resistant cells. It was possible to maintain the phagemid in L-broth supplemented with ampicillin, but as soon as selective pressure was relaxed the phagemid was rapidly lost. pVET4m was stable at low growth rates but lost at higher rates and pVET4n was almost as unstable as pORF1. Both of these phagemids could be stabilised when grown with ampicillin in the media (see Table 7.1, B.)

The pTrc99A based plasmids, pNIK1m and pNIK1n were shown to be stable even when grown in a shaken flask, but because of the presence of *lacI*^{q} on the plasmid, they would not be expressing PA. Thus they were also grown with IPTG induction, with and without ampicillin selection, to see if the plasmids became unstable when producing PA. As can be seen in Table 7.1, B. the plasmids remained stable under all growth conditions. This was surprising as it had previously been noted that pNIK1n was very unstable without

Table 7.1 Stability of constructs

A. % = the percentage still ampicillin resistant.

Abs = the final Abs_{600} after overnight culture.

ND = not done

S. typhimurium SL 3261 containing various plasmids was grown overnight in L-broth in one of three ways: statically in 10 ml in a sealed Universal, shaking in 10 ml in a sealed Universal, or shaking in 50 ml in a loosely sealed flask. The Abs_{600} was measured and aliquots diluted in PBS to give approximately 10^4 cells / ml. 50 μl of these dilutions was plated out using a spiral plater on both L-agar and L-agar supplemented with ampicillin and the plates incubated overnight. The colonies present were scanned with a laser counter and the count computed using BEN software. The ratio of colonies on the plates was used to find the percentage that were still ampicillin resistant.

B. In a similar experiment four of the constructs were cultured in either L-broth, L-broth+Amp, L-broth+IPTG or L-broth+Amp+IPTG. The cultures were either grown statically in 10 ml in a sealed Universal or in 50 ml in a shaken flask. Only % still ampicillin resistant are shown.

'ND' = not done as these plasmids are constitutively expressed in *S. typhimurium*.

C. *S. typhimurium* SL 3261 containing pNIK1n plasmids designated C1 or E5 were grown as described above in shaken flasks containing either 50 ml of L-broth or L-broth+IPTG. The % still ampicillin resistant is shown

A.

S. typhimurium SL 3261 containing	Static Universal		Shaken Universal		Shaken Flask	
	%	Abs	%	Abs	%	Abs
pBluescript II	95	0.34	90	0.93	95	2.4
pPA26	60	0.42	30	0.98	30	4.35
pCB1	100	0.36	95	0.83	100	3.2
pORF1	1	0.39	ND	ND	ND	ND
pVET4m	100	0.41	10	0.97	1	3.6
pVET4n	10	0.47	5	0.97	1	3.85
pNIK1m	100	0.47	90	0.9	85	4.1
pNIK1n	90	0.43	85	0.9	80	3.8
pSIG13	25	0.32	10	0.95	5	3.4

B.

S. typhimurium SL 3261 containing	L-broth		L-broth + Amp		L-broth + IPTG		L-broth + Amp + IPTG	
	Static	Shaken	Static	Shaken	Static	Shaken	Static	Shake
pVET4m	100	1	90	70	ND	ND	ND	ND
pVET4n	10	1	90	80	ND	ND	ND	ND
pNIK1m	100	85	100	90	90	100	100	100
pNIK1n	90	80	90	80	90	90	95	100

C.

S. typhimurium SL 3261 containing	L-broth	L-broth + IPTG
pNIK1n C1	65	5
pNIK1n E5	100	90

Table 7.1 Stability of constructs

antibiotic selection (Section 5.5.1). The possibility arose that by plating the original construct on ampicillin selection plates, to make the original starter culture, this had selected a mutation which stabilised the plasmid. To investigate this, the original -70°C stock of *S. typhimurium* SL 3261 / pNIK1n (designated C1) was compared to the -70°C stock made from the ampicillin selection plate as described above (designated E5). The results are shown in Table 7.1, C. This shows that the two cultures behaved differently, with the original culture (C1) being more unstable, especially when induced. A Western blot showed that pNIK1n E5 still expressed PA with the typical banding seen with pNIK1n C1.

The pUC19 based plasmid pSIG13 behaves in a similarly unstable manner to the pBluescript phagemids with the 63 kDa insert. pBluescript II has the same origin of replication as the pUC plasmids.

As plasmid stability, without antibiotic selection, was a problem with many of the clones, a method to stabilise the plasmids was investigated.

7.2.2 Mechanisms of plasmid instability

Plasmids are lost (and hence unstable) when bacteria divide and produce a high incidence of daughter cells without the plasmid. Low and intermediate copy number plasmids have specific active mechanisms to ensure stable inheritance of the plasmids such as the *par* region in the *S. typhimurium* virulence plasmid (Tinge & Curtiss, 1990). This region can stabilise the segregation of low to moderate copy number plasmids but was deleterious to cells containing the locus on a high copy number pUC18 clone (Tinge & Curtiss, 1990).

The naturally high copy number plasmid ColE1 is very stably maintained but without an analogous *par* region. Various models for the stability of high copy number plasmids have been suggested including purely random models where the likelihood of a daughter cell not containing at least one of the many plasmid copies is remote (Summers & Sherratt, 1984). However, it has been found that the commonly used cloning vectors derived from ColE1 are lost from cultures at frequencies between 10^{-2} and 10^{-5} per cell per generation (Summers & Sherratt, 1984). This is now known to be due to the formation of multimers of these plasmids which can randomly partition into only one daughter cell. During the construction of the commonly used cloning vectors a region of ColE1, called the *cer* locus, has been lost. This region is necessary for a recombination event that converts multimers

to monomers and so increases the chances that the plasmid copies will segregate evenly between the daughter cells. The *cer* region can be inserted back into cloning vectors and sometimes increase their stability (Summers & Sherratt, 1984; Charles & Dougan, 1990).

The *cer* locus therefore offered the possibility of stabilising the plasmids. pORF1 was selected as a model because it produces the full-length PA which seems from Western blots to be fairly stable.

7.2.2.1 Construction of phagemid pORF1c

pORF1c was constructed by cloning the *cer* region into pORF1. The *cer* locus was available on the plasmid pKS492 (Prof. DJ Sherratt, University of Glasgow). This plasmid was constructed by cloning the 279 bp *Hpa*II-*Taq*I *cer* fragment from ColE1 into the *Acc*I site of pUC9 to form plasmid pKS490. The *Bam*HI-*Pst*I fragment from pKS490 was cloned in to the *Bam*HI-*Pst*I sites of pUC18 to form plasmid pKS492.

The *cer* locus can be expected to function when it is cloned into any non-essential region of the plasmid. This can be accomplished by cloning the locus in the unique restriction sites downstream of the coding region of the expressed gene. The *cer* locus does not need to be in a specific orientation. Phagemid pORF1 was constructed by cloning a PCR product into the *Kpn*I-*Bam*HI sites of pBluescript II KS+. This removes many sites from the polylinker and leaves few unique sites downstream from the PA gene insert. After checking the sites available it was found that the *cer* locus could not be directly cloned into pORF1. A strategy was devised to sub-clone a *Bam*HI-*Hin*dIII *cer* fragment from pKS492 into *Bam*HI-*Hin*dIII cut pACYC184. Then a *Bam*HI-*Xba*I fragment can be cloned from the pACYC184/*cer* construct into *Bam*HI-*Xba*I cut pORF1. This positions the *cer* locus approximately 135 bp downstream of the PA ORF stop codon.

The phagemid pORF1c was constructed and sequenced over the region of the *cer* insertion to verify that the construct was correct.

7.2.2.2 Stability of pORF1c

When pORF1c was grown in 10 ml L-broth in a shaken Universal tube, no amp^r colonies could be found after an overnight culture. When grown in 10 ml L-broth supplemented with ampicillin in a shaken Universal tube the cultures grew slowly only

reaching an Abs_{600} of 0.3 overnight rather than the expected 1.0 (see Table 7.1 A) and only 20-40% of the cells grew on L-agar plates supplemented with ampicillin.

7.3 Growth curves and expression

Growth curves were determined by growing an overnight culture of the appropriate organisms in L-broth supplemented with ampicillin. The Abs_{600} was used to estimate the cell density and then equal numbers of cells were used to seed 100 ml of L-broth supplemented with ampicillin. This culture was grown at 37°C with shaking and the Abs_{600} determined every hour until 10 hours and then at 24 hours. The log of the Abs_{600} was plotted against time. Aliquots of the culture were also removed each hour and used to make whole cell lysates. From the Abs_{600} readings the cell density was calculated and a known number of cells were run on SDS-PAGE and were subsequently Western blotted.

With the unstable clones it was found that as the cell density increased the ampicillin in the medium was depleted by β-lactamase activity and this allowed plasmid free segregants to arise. This invalidated an analysis of the growth curves as they did not represent the growth of plasmid-containing cells at all time points. Growth curves for the more stable clones are described below.

7.3.1 Growth curves and expression results

Growth curves for *S. typhimurium* SL 3261 alone or with phagemids pBluescript, pVET4m or pVET4n, are shown in Figure 7.1. *S. typhimurium* SL 3261 alone was able to grow faster than bacteria containing a phagemid. It was interesting to note that pBluescript caused a similar slowing of growth to phagemids which also were expressing PA. This suggests that PA expression is not responsible for the slower growth and has no additional effect on growth. This was unexpected as it was presumed that the increased instability of these PA producing phagemids over pBluescript (Table 7.1, A.) was related to a toxic or metabolic load effect of PA expression on the bacteria and would be reflected by a slower growth rate.

The amount of PA present in *S. typhimurium* / pVET4m and pVET4n was shown to be at approximately a constant level from 4 to 7 hours but was lower at 24 hours (results not shown). This might reflect degradation of the PA during the static growth phase.

Figure 7.1 Growth curves for *S. typhimurium* SL 3261 alone, and with phagemids pBluescript, pVET4m and pVET4n

Starter cultures were grown overnight from -70°C stocks in L-broth supplemented with ampicillin except for the *S. typhimurium* SL 3261 cultures. The Abs_{600} was used to estimate cell densities and the same number of cells added to flasks containing 100 ml of L-broth with ampicillin if required. The flasks were grown at 37°C in a shaking incubator and aliquots removed every hour for Abs_{600} readings (0-10 hours and at 24 hours). If the Abs_{600} reading was greater than 1, a ten-fold dilution in L-broth was measured and the Abs_{600} reading multiplied by 10. The log values of the Abs_{600} readings were plotted against time.

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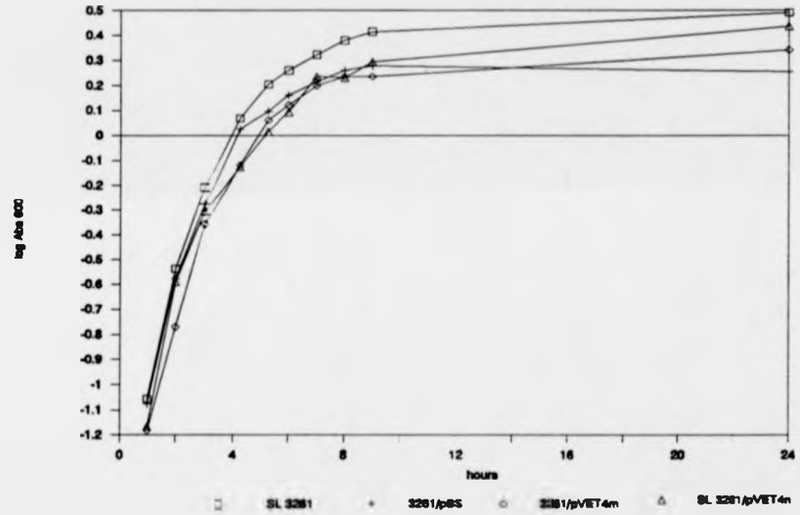

Figure 7.1 Growth curves for *S. typhimurium* SL 3261 alone, and with phagemids pBluescript, pVET4m and pVET4n

7.4 Animal experiments

A preliminary animal experiment was carried out at CAMR in collaboration with Dr. P.C.B. Turnbull. Five groups of 8-10 week old female BALB/c mice were used.

To test the effectiveness of *aro S. typhimurium* SL 3261 in protecting against homologous wild-type *S. typhimurium* SL 1344 challenge, a control group was given 0.2 ml PBS by intragastric inoculation and another group given 0.2 ml of a suspension of *S. typhimurium* SL 3261 (approximately 10^9 cells). Both groups were challenged 21 days later with 0.2 ml of a suspension of *S. typhimurium* SL 1344 (approximately $4x10^8$ cells) which is equivalent to 250 LD_{50} (O'Callaghan *et al.*, 1988).

The other three groups were intragastrically inoculated with 0.2 ml (approximately 10^9 cells) of preparations of *S. typhimurium* SL 3261 containing either pPA26, pVET4m or pSIG13 on days 1 and 14. Sera from these groups were obtained at day 28 and tested by both ELISA and Western blotting for the presence of antibodies to PA, and by Western blotting for antibodies against *S. typhimurium* antigens. It was not possible to challenge the animals with *B. anthracis* in this experiment.

Positive anti-PA control serum was produced by inoculating one mouse with a mixture of the human PA vaccine supplemented with additional purified PA (50 ng PA in 0.65 ml vaccine). Six 100 μl doses of the mixture were given S/C on days 1, 2 , 3, 4, 7, 9 and sera collected on day 14.

7.4.1 Results of animal experiment

The experiment to test the effectiveness of vaccination against challenge with *S. typhimurium* SL 1344 had to be prematurely terminated eight days after challenge. At this point only 2/11 of the control group had survived but 10/12 of the vaccinated group were alive. This was disappointing in that the vaccinated group should have been solidly protected. This may reflect inexperience in the intragastric inoculation procedure leading to a variation in the amount each mouse was inoculated with.

The anti-PA inhibition ELISA was carried out by Dr. P.C.B. Turnbull at CAMR and failed to show any anti-PA antibodies except in the positive control sera.

Western blotting showed that all groups vaccinated with *S. typhimurium* SL 3261 had produced antibodies against *Salmonella* antigens, but only the positive control sera contained

antibodies against PA. Blots for the control groups and *S. typhimurium* SL 3261 / pVET4m are shown in Figure 7.2. Sera from animals vaccinated with *S. typhimurium* SL 3261 containing pPA26 and pSIG13 produced similar results to pVET4m (results not shown).

Therefore it was concluded that the *S. typhimurium* recombinants had induced an immune response against the *Salmonella* antigens, but using only the anti-PA antibody ELISA and Western blotting a response against the PA could not be detected.

7.5 Conclusions

Assuming that *in vitro* experiments can predict *in vivo* results, there is not an obvious recombinant that is ideal for *in vivo* evaluation. The use of the pUC plasmid based phagemid pBluescript as the vector in many constructs may have created some problems. Although the phagemid is relatively stable (90-95%) it appears to have an inhibitory affect on growth rate compared to *S. typhimurium* SL 3261 alone (Figure 7.1). When PA is expressed at low levels in pBluescript (pCB1) the stability is unaffected (95-100%) but as expression of PA is increased (pORF1) the stability is greatly reduced (1%). The level of expression of PA from pCB1 is low and for this reason it is not considered suitable as a vaccine candidate. pPA26 expresses less PA than pCB1 and is also less stable. Expression of PA-63 (pVET4m and n) gives intermediate stability (ranging from 100% to 1% at different growth rates) but does not further inhibit growth in addition to pBluescript's effect. Stability was difficult to determine when the phagemids were able, in the later stages of growth, to evade the ampicillin selection pressure by presumably depleting the antibiotic concentration by β-lactamase production. It therefore appears that pBluescript alone can affect the growth rate and that high expression of PA can diminish stability.

An attempt was made to stabilise the phagemid pORF1 by inserting the *cer* locus after the PA ORF. This could not be shown to enhance the phagemid's stability and appeared to further inhibit growth when antibiotic selection pressure was applied although, by 12 hours, this selection pressure was evaded and plasmid free segregants were found. This approach to stabilising the plasmids was not pursued.

The pNIK plasmids were stable but did not produce a large amount of full-length product and they had an undefined character. It appears that some change has occurred that affects plasmid stability, but without preventing PA production. The isolation of this mutation probably reflects the unstable nature of pNIK1n C1. As it was produced by

Figure 7.2 Western blot with animal sera showing antibodies against *Salmonella* antigens and PA

Control (negative) sera was from mice orally administered PBS (labelled 'Control'). Two other groups of mice were orally inoculated with *S. typhimurium* SL 3261 alone (labelled 'SL 3261') or containing phagemid pVET4m (labelled 'SL 3261/pVET4'). Sera from mice in each group were pooled and 60 μl diluted in 4 ml PBS. One mouse was vaccinated with PA, as described in the text, and 10 μl of this sera was mixed with 4 ml PBS (labelled 'PA vaccinated').

S. typhimurium SL 3261, with and without phagemid pVET4m, were used to make whole cell lysates for use as negative and positive controls for PA production and as positive controls for Salmonella antigens. Purified PA (83 kDa) was used as another positive control for PA. Bio-Rad prestained MW markers were run on each gel, the apparent MWs of the top four markers are 92 kDa, 66 kDa, 45 kDa and 29 kDa. Samples were separated by SDS PAGE on 8% gels and blotted onto PVDF membrane. Four strips containing the same antigen lanes were incubated in the four different diluted animal sera. Antibodies bound to the strips were detected with HRP linked anti-mouse IgG antibodies and developed using DAB.

The control mice did not have antibodies against either Salmonella antigens or PA. The PA vaccinated mouse serum contained antibodies against both 63 kDa PA expressed by *S. typhimurium* (lane 2, PA vaccinated) and purified PA (lane 3, PA vaccinated) but did not have antibodies against Salmonella antigens (lane 1, PA vaccinated). The group vaccinated with *S. typhimurium* SL 3261 only had anti-Salmonella antigens (lanes 1 and 2, SL 3261), as did the group vaccinated with the *S. typhimurium* containing pVET4m (lanes 1 and 2, SL 3261/pVET4). This suggested that the PA expressed by the *S. typhimurium* SL 3261/pVET4m had not induced an anti-PA antibody response (no bands in lane 3, SL 3261/pVET4).

Lane 1 Whole cell lysate of *S. typhimurium* SL 3261

Lane 2 Whole cell lysate of *S. typhimurium* SL 3261 / pVET4m

Lane 3 Purified PA (83 kDa)

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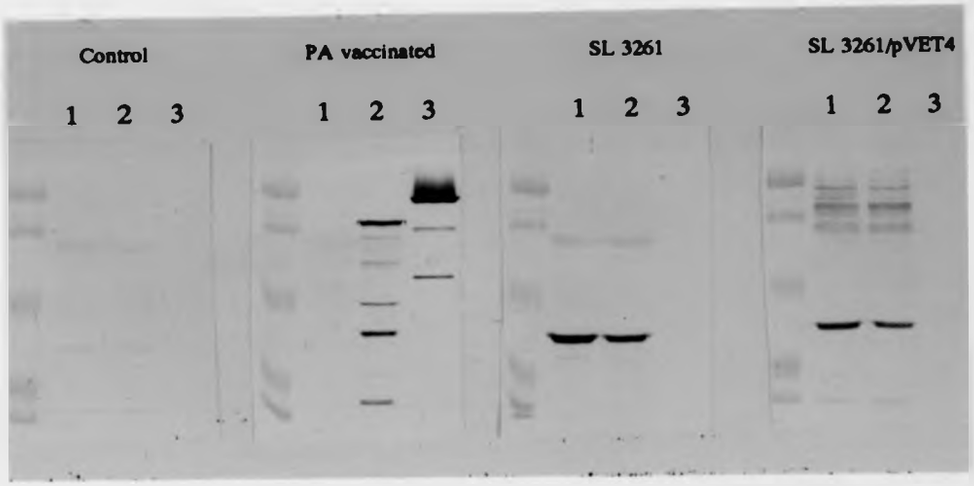

Figure 7.2 Western blot with animal sera showing antibodies against *Salmonella* antigens and PA

selecting on an ampicillin plate, without IPTG, it appears that the instability is not related to expression of PA but to some consequence of the plasmid sequence. As pNIK1m does not show the same instability two possibilities are apparent. Firstly, pNIK1m may have mutated in the same manner as pNIK1n E5, but at an earlier stage before the unstable construct was identified. Secondly, the sequence differences between pNIK1m and pNIK1n may be responsible for the different stabilities. The two plasmids only have known sequence differences at the C-terminal of the PA ORF with pNIK1m having a 25 bp region which differs to a 48 bp region in pNIK1n. If this region was responsible for the instability, a mutation could have occurred in this region in pNIK1n E5. Sequencing over this region would be possible, but even if a sequence change between pNIK1n C1 and E5 could be found, it would not prove that such a change is responsible for the stability differences as other unidentified changes may be present elsewhere on the plasmid. Plasmid stability is more likely to be affected by mutations in the regulatory regions of the plasmid. Before these plasmids could be used for *in vivo* evaluation, it would be necessary to inactivate the *lacI*^{q} gene and the constitutive expression of PA might adversely affect stability.

As pSIG13 does not export the 63 kDa fragment as expected it would not seem to have any particular advantage over the pVET4 phagemids.

The animal experiment at CAMR showed that oral challenge with the constructs tested did not induce a detectable antibody response. This suggests that either other constructs or a different method of administration is needed to generate an immune response. The mice responded to the *S. typhimurium* antigens suggesting that the vaccine strains were able to colonise and persist as expected.

The aim of the animal challenge experiments is to show if *S. typhimurium* expressing PA can induce protective responses against challenge with *B. anthracis*. As it is known that full-length purified PA can induce protective responses when administered with adjuvants it seems logical to choose a construct expressing full-length PA. pORF1 is unstable, but for the purposes of this experiment, it might be stabilised by the administration of antibiotics. Antibiotic treatment has been shown to stabilise plasmids which otherwise are rapidly lost *in vivo* (Tarkka *et al.*, 1989; Molina & Parker, 1990). Therefore pORF1 was selected as a recombinant for use in the animal study. Also, it was decided to evaluate pVET4m alongside pORF1. pVET4m was also unstable and so would be administered with antibiotic.

It produces a product which varies in both size and cellular location to pORF1 PA and so would act as a useful comparison to pORF1.

CHAPTER 8

Testing of candidate *S. typhimurium* recombinants in animals

8.1 Introduction

Having selected *S. typhimurium* SL 3261 / pORF1 and *S. typhimurium* SL 3261 / pVET4m as the candidate recombinants for animal testing, experiments were designed to evaluate their protective potential. Neither of these recombinants were ideal as a candidate vaccine, but it was hoped to show that PA delivered to the immune system in *S. typhimurium* could induce protective immune responses. If the principle of vaccination using this system can be established then further work to stably express PA in *S. typhimurium* would be justified.

Firstly, it was necessary to confirm that mice vaccinated with an *aro S. typhimurium* could be successfully protected from wild type *S. typhimurium* challenge. This would show that the culturing, storage and inoculation procedures were effective. Secondly, as it was known that the PA expressing constructs were unstable *in vitro*, a strategy was devised to enhance the possibility of an immune response to the PA. Suitable positive and negative controls were included. Although serum anti-PA antibodies would indicate an immune response, it is known that they are not necessarily predictive of protection. There is no immunological test which is known to correlate with protection and so the vaccinated mice were challenged with *B. anthracis* in order to assess protective immunity.

8.2 Establishing the animal model

The animal model and vaccination techniques were established using two groups of 10 BALB/c female mice aged 8-10 weeks. Prior to inoculations, the animals were lightly anaesthetized with halothane. All inoculations were given into the lower oesophagus (referred to as an 'intragastric inoculation') using a blunt-ended needle. The control group of animals was administered two doses of 0.2 ml of PBS. 3 days apart. The other group was given 0.2 ml PBS containing $6x10^9$ viable *S. typhimurium* SL 3261 and 3 days later a second inoculation containing $4x10^9$ *S. typhimurium* SL 3261. Viable counts were obtained retrospectively by serial dilutions of the bacterial suspension and plating on appropriate media.

After 41 days both groups of mice were challenged with 0.2 ml of *S. typhimurium* SL 1344 resuspended in PBS, administered intragastrically. Assuming that the intragastric LD_{50} for BALB/c mice is 1.6×10^8 bacteria (O'Callaghan *et al.*, 1988), serial dilutions and platings showed that the dose given contained 10^8 viable bacteria and so was equivalent to

63xLD_{50}. This challenge dose was smaller than the 250 LD_{50} intended. The mice were monitored for 14 days. Moribund animals were humanely euthanised and deaths were recorded twice a day.

4 of the ten control animals died 6-7 days after challenge. None of the vaccinated group died.

8.3 Mouse protection experiment

As the phagemid constructs are known to be unstable *in vitro*, two strategies were used to increase the possibility of inducing an immune response to the PA. Firstly, the IV route was used for administration of the *S. typhimurium* strains expressing PA as this delivers the bacteria to the reticulo-endothelial system by a more direct route and has given immune responses when oral administration has not (pers. comm. Prof. G. Dougan). Secondly, ampicillin was administered to some groups to stabilise the plasmid. This method has been reported by Tarkka *et al.* (1989) when 20 mg of ampicillin administered IP once a day was shown to stabilise an unstable plasmid. In preliminary experiments this dose was found to debilitate the mice and so the regimen used was to administer 7.5 mg ampicillin (50 μl of 150 mg/ml 'Penbriten' injectable) SC once a day for 7 days. With this regimen some mice developed soft swellings at the site of injection by the end of the 7 day administration period but otherwise remained healthy. The efficacy of this treatment in stabilising the constructs was assessed in suitable control groups.

The experimental groups are summarised in Table 8.1 and described below.

8.3.1 Group A - Negative controls

Group A were administered two IV doses of $5x10^5$ *S. typhimurium* SL 3261 14 days apart and challenged with *B. anthracis* 45 days later. This period of time should allow clearance of the *S. typhimurium* from the mice and also allow non-specific immune responses to wane. Strugnell *et al.* (1992) with a P.69 antigen of *Bordetella pertussis* model showed that non-specific immunity affect protection at day 14 but not at day 28.

8.3.2 Groups B and C - Positive controls

It is known that vaccination of mice with PA mixed with adjuvant can protect against challenge with *B. anthracis* (Ivins *et al.*, 1992). There is however great variation in

Table 8.1 Mouse protection experiment - Groups

8-10 week old female BALB/c mice were assigned to one of ten groups designated by the letters A through J. Further details of the treatments given to each group are contained in the text.

PA = Protective Antigen (from *B. anthracis*)

rPA = recombinant PA purified from *E. coli* / pORF1

FIA = Freunds Incomplete Adjuvant

IV = intravenous

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Group	Summary	No. mice	Procedures
A	Negative controls, *S. typhimurium* SL 3261	8	2 IV doses days 1 and 14
B	PA vaccinated	8	3 doses IP PA + FIA (10-14 days apart)
C	rPA vaccinated	8	3 doses IP rPA + FIA (10-14 days apart)
D	*S. typhimurium* SL 3261 / pORF1 + ampicillin	12	2 doses IV days 1 and 14. Ampicillin days 1-7 and 14-21
E	*S. typhimurium* SL 3261 / pORF1	12	2 doses IV days 1 and 14
F	*S. typhimurium* SL 3261 / pVET4m + ampicillin	12	2 doses IV days 1 and 14. Ampicillin days 1-7 and 14-21
G	Stability after IV *S. typhimurium* SL 3261 /pORF1 + ampicillin	10	one dose IV and euthanise 3 mice on days 3, 8 and 14; with ampicillin days 1-7.
H	Stability after IV *S. typhimurium* SL 3261 / pORF1 without ampicillin	10	one dose IV and euthanise 3 mice on days 3, 8 and 14.
I	Stability after IV *S. typhimurium* SL 3261 / pVET4m + ampicillin	10	one dose IV and euthanise 3 mice on days 3, 8 and 14; with ampicillin days 1-7.
J	Stability after IV *S. typhimurium* SL 3261	10	one dose IV and euthanise 3 mice on days 3, 8 and 14.

Table 8.1 Mouse protection experiment - Groups

the susceptibility of different mice types to different strains of *B. anthracis* (Welkos *et al.*, 1986) and the adjuvant used can markedly alter the protective response (Ivins *et al.*, 1992). It was not possible to find a literature report of the amount of purified PA mixed with Freunds Incomplete Adjuvant (FIA) that would protect BALB/c mice against a specific *B. anthracis* Vollum challenge. From discussions with Dr ED Williamson (CBDE) it was decided to use an amount of PA/FIA that was known to produce a strong antibody response in BALB/c mice. Therefore Group B was inoculated with 3 IP doses of 10 μg PA (purified from *B. anthracis*) mixed 50:50 with FIA, given 14 days apart. The PA expressed by the recombinant clones should be immunologically identical to the PA purified from *B. anthracis*. To verify this, recombinant PA (rPA) was purified from the periplasm of *E. coli* containing phagemid pORF1. Group C were vaccinated in an identical manner to Group B but using 10 μg of rPA. Both groups were challenged with *B. anthracis* 18 days after the third vaccination.

The rPA was purified from the periplasm of *E. coli* JM 109 / pORF1. *E. coli* were used as, with IPTG induction, higher amounts of rPA could be produced. By comparing bands on a Western blot with known amounts of PA, it was estimated that 7-12 mg of rPA could be recovered from one litre of culture medium. This equated to recovery of about 85% of the rPA released from the cells by the lysozyme/EGTA treatment with 13% being left in solution after the ammonium sulphate precipitation step, and 2% being lost in the ultracentrifugation step to remove LPS. The total expressed was therefore about 8-14 mg rPA/litre of induced culture.

8.3.3 Groups D, E and F - Vaccinated groups

In a similar manner to Group A, mice were inoculated IV on days 1 and 14 with $5x10^5$ *S. typhimurium* containing either pORF1 or pVET4m. Inoculation doses were prepared as described in Chapter 2 from seed stocks of bacteria which had their *aro* and smooth LPS phenotypes confirmed. Groups D and E were inoculated with bacteria containing pORF1, and Group F was inoculated with pVET4m. Ampicillin was administered to Groups D and F. Mice were given 7.5 μg ampicillin SC for two periods of seven days starting 4 hours before each of the IV inoculations. Space limitations prohibited the use of a non-ampicillin treated pVET4m group.

8.3.4 Groups G, H, I and J - Stability *in vivo*

In order to assess the effect of ampicillin in stabilising the plasmids *in vivo*, Groups G, H and I were inoculated once with *S. typhimurium* containing the plasmids to parallel Groups D, E and F. Ampicillin was administered to Groups G and I (to parallel Groups D and F) for 7 days. Group J was inoculated with *S. typhimurium* SL 3261 without a construct to act as a control group. Three mice from each group were euthanised on days 3, 8 and 14 and the livers and spleens removed and organ counts determined. At day 14, 10 ampicillin resistant colonies from Groups G and I were picked, grown overnight in L-broth supplemented with ampicillin and Western blotted to check for expression.

The organ counts for the *S. typhimurium* recombinants were lower than expected ($10\text{-}10^2$ instead of $10^5\text{-}10^6$, (O'Callaghan *et al.*, 1988)) and so few colonies were counted and the variation between the three mice at each sampling point was large. The data is presented as means without standard errors so that the trends can be identified. The mean total organ counts are shown in Figure 8.1. The percentage which were ampicillin resistant (amp^r) at each time point are shown below:

Percentage of total organ count still ampicillin resistant						
Day	Group G.		Group H.		Group I.	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
3	30	70	10	30	100	100
8	70	40	1	2	80	80
14	40	30	0	0	20	20

At day 14, 9 out of 10 amp^r clones in group G and 8 of 10 in Group I were still expressing PA.

From Figure 8.1 it can be seen that all the constructs (Groups G, H and I) have lower organ counts than the *S. typhimurium* SL 3261 control group (Group J) by a factor of 10^2 to 10^3. The Group H count appears to be increasing faster than the other ampicillin treated groups and this may be correlated with the loss of amp^r (and by implication the plasmid) in this group. The ampicillin treatment seems to have increased the percentage of

Figure 8.1 Liver and Spleen counts for Groups G, H, I, and J.

Groups G, H, I and J were inoculated with $5x10^5$ cfu on day 0 as shown below.

Group	No. in Group	Vaccinated IV with:
G	10	*S. typhimurium* SL 3261 / pORF1 + ampicillin
H	10	*S. typhimurium* SL 3261 / pORF1
I	10	*S. typhimurium* SL 3261 / pVET4m + ampicillin
J	10	*S. typhimurium* SL 3261

Ampicillin was given to Groups G and I between day 0 and 7.

On days 3, 8 and 14 three mice in Groups G, H, I and J were euthanised and their livers and spleens removed aseptically and placed in ice-cold PBS. The organs were homogenised in 10 ml of PBS for 2 minutes in a Stomacher. Aliquots of the homogenised organs were plated on L-agar plates and L-agar plates supplemented with ampicillin (except for Group J). The plates were incubated overnight at 37°C and the colonies on each plate counted. From the plate counts the total organ count was determined. The log of the total organ count was plotted against time.

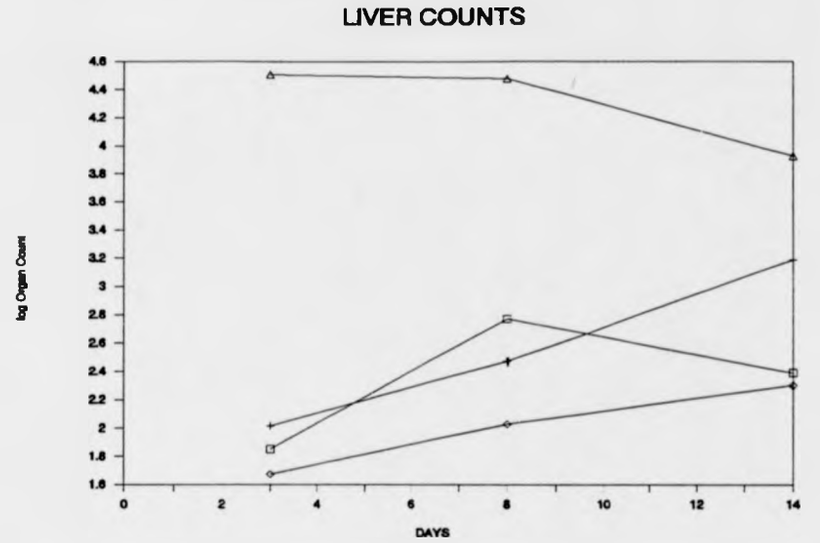

Figure 8.1 Liver and Spleen counts for Groups G, H, I, and J.

amp^r bacteria present. If Groups G and H are compared, Group G has 40-70% of colonies amp^r at day 8, but only 1-2% of the non-ampicillin treated Group H are amp^r. Group I parallels the ampicillin treated group G, although there is no specific matched control group. The majority of the amp^r colonies are still expressing PA.

It was possible that the lower colonization rates seen could be the result of the presence of the phagemid vector pBluescript. To test if this phagemid alone caused lower colonization, 4 mice were inoculated IV with 4 x 10^5 *S. typhimurium* SL 3261 / pBluescript II. They were not given ampicillin. After 7 days they were euthanised and total organ counts of both amp^r and amp^s *S. typhimurium* determined. The mean liver count was 1.95 x 10^3 and the mean spleen count was 2.95 x 10^3. By day 7 all the bacteria had become ampicillin sensitive.

From Figure 8.1 it can be seen that this colonisation level lies above the PA expressing clones but falls short of the *S. typhimurium* without a phagemid. This result suggests that either the presence of pBluescript can cause a decrease in colonization or that the procedure of electroporating the bacteria and selecting for recombinants has affected their ability to colonise. For instance electroporation and *in vitro* growth on agar plates could select for LPS mutants with reduced virulence, although the smooth phenotype of the inoculating cultures was confirmed before use. All the bacteria recovered at day 7 were amp^s which suggests that the *in vivo* instability of the PA expressing constructs may be due to the phagemid vector. This also indicates that the *in vitro* results showing that pBluescript was stable (Table 7.1) were not predictive of *in vivo* results.

8.4 Antibody response

2 days before the challenge with *B. anthracis*, all mice in Groups A through F were tail bled and their sera pooled by groups. The level of anti-PA antibodies was determined by ELISA. Plates were coated with PA and the pooled serum double diluted across the plate starting at an initial dilution of 1/10. Anti-PA antibody was detected with HRP conjugated sheep anti-mouse IgG antibody developed with ABTS. Only groups B and C had detectable levels of anti-PA antibodies, and these groups had high titres of 1/5,120 and 1/10,240 respectively.

Each group's pooled sera were also used to probe Western blot strips containing 3 lanes of MW markers, PA and a whole cell lysate of *S. typhimurium* SL 3261 (results not

shown). Groups B and C reacted with the PA as expected. Group C also reacted with proteins in the *S. typhimurium* SL 3261 lane, possibly due to cross-reacting antibodies generated to the *E. coli* proteins contaminating the rPA preparation used in the group C immunisations. Groups D, E and F reacted strongly with the *S. typhimurium* SL 3261 lane with a smeared appearance. This may be due to antibodies reacting with lipopolysaccharide. The serum from these three groups reacted faintly with PA suggesting that a low level of anti-PA antibodies might be present, but presumably this level was below the detection limit of the ELISA.

8.5 Protective responses

The mice in Groups A through F were challenged with *B. anthracis*. The LD_{50} for *B. anthracis* Vollum 1B spores injected SC in BALB/cJ mice (Jackson Laboratory strain of BALB/c mice) has been given as 6.6 spores (Welkos *et al.*, 1986). It was assumed that BALB/c mice would behave in a similar manner. From previous experience at CBDE, it was expected that 10-20 LD_{50} should lead to the death of all susceptible mice. A preliminary experiment was carried out to verify this.

8.5.1 Determination of *B. anthracis* challenge dose

B. anthracis Vollum 1B spore stock was prepared as described and stored at 4°C. To try to evaluate what the challenge dose would be in mice of a similar weight to that of the vaccinated mice at the time of their challenge (8-10 weeks at the start and challenged 8 weeks later, therefore age 16-18 weeks at challenge), 28 older mice were ordered. The only older mice that were available were ex-breeders which were potentially much older. 8 of these mice were challenged SC with 0.25 ml of a diluted spore preparation which was subsequently determined to contain 341 cfu/ml. Thus the dose given was 85 cfu/mouse. This equates to approximately 13 LD_{50}. All of the mice died between 3½ and 6 days after challenge with a mean time to death (ttd) of 5.4 days. This is shown graphically in Figure 8.2.

The time to death was similar to the 4.1-6.7 days described by Welkos *et al.*, (1986) for a dose of 60 spores. Two other doses were also evaluated; 10 mice were challenged with 0.125 ml (38 cfu, approximately equivalent to 6 LD_{50}) and another 10 mice with 0.5 ml (150 cfu, approximately equivalent to 23 LD_{50}). In the low dose group two mice survived until

Figure 8.2 Time to death of mice challenged with three doses of *B. anthracis* Vollum spores.

Old breeder BALB/c mice were obtained as the nearest approximation to the weight that the vaccinated mice would have achieved by the time of the *B. anthracis* challenge. 8 mice were given 85 cfu SC (0.25 ml of a diluted *B. anthracis* Vollum 1B spore preparation containing 341 cfu/ml) equivalent to 13 LD_{50} (Welkos *et al.*, 1986). Subsequently, two groups of 10 mice were given 38 and 150 cfu SC (0.125 ml and 0.5 ml of a preparation containing 300 cfu/ml) equivalent to 6 LD_{50} and 26 LD_{50}. Deaths were recorded every 12 hours and are plotted against time, thus for each day there are AM and PM data points.

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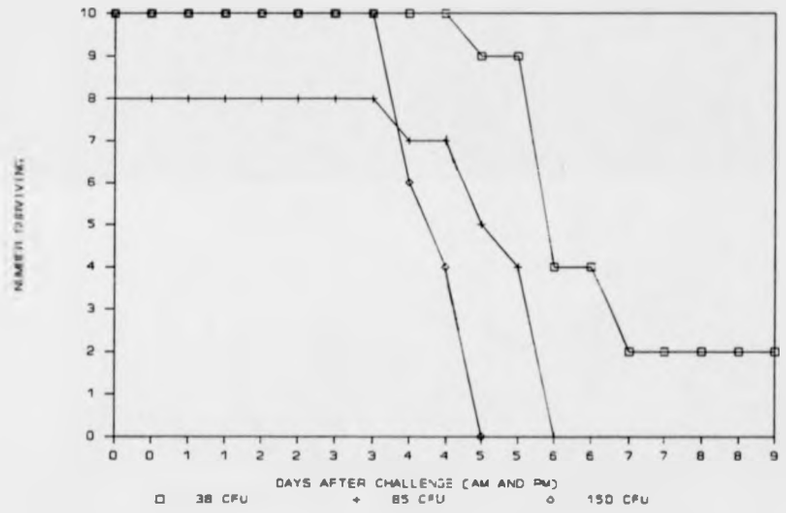

Figure 8.2 Time to death of mice challenged with three doses of *B. anthracis* Vollum spores

day nine when the experiment was terminated. In the high dose group, all the animals died with a time to death approximately 24 hours less than for the middle dose group. From this result, it was concluded that the dose required to kill all mice was between 38 and 85 cfu. It was decided to challenge the vaccinated and control mice with approximately 85 cfu.

8.5.2 Challenge of Groups A through F with *B. anthracis*

Mice in groups A through F were challenged with a SC injection of 0.25 ml of a *B. anthracis* Vollum 1B spore preparation (369 cfu/ml). Thus each mouse was administered approximately 92 cfu (14 LD_{50}). The percentage of mice surviving at each 12 hour interval after challenge is shown in the graph in Figure 8.3. The table in Figure 8.3 shows the mean time to death (ttd) for those animals dying in each Group and the percentage that died in each Group. As a protective response may be indicated by either animals surviving the challenge or an increase in the ttd, a protection index (PI) was derived that takes both factors into account. This data is also presented as separate group histograms of deaths occurring in each 12 hour period between days 3 and 8 after challenge in Figure 8.4.

Group A all died between 4 and 6 days after challenge with a mean time to death of 4.6 days and a PI of 4.6. The ttd was less than the 5.4 day ttd of the group given 13 LD_{50} to determine the challenge dose. This may be due to the older ex-breeder mice used in the determination of challenge dose experiment having an age related immunity.

In Group B, 2 out of 8 animals (25%) survived with 6 deaths occurring between days 4 and 7. The ttd was 5.2 and the PI was increased to 6.9.

In Group C, 3 out of 8 animals (37.5%) survived with deaths between 3 and 6 days. Some animals in this group died earlier than other groups, but only by a maximum of 24 hours. The ttd was 4.5 and the PI 7.2. Groups B and C give a similar picture of deaths and partial protection which suggests that the PA and rPA behave in a similar manner in inducing the same level of protective immunity.

In Group D, 3 out of 10 mice (30%) survived showing that this vaccine was able to protect some animals. Deaths occurred from 4 to 8 days with a ttd of 6.2 and a PI of 8.9. This Group had the highest PI of the Groups. The increased ttd may represent a partially protective immune response which subsequently became overwhelmed.

In group E, all 10 animals died with a mean ttd of 5.5 days and a PI of 5.5, one greater than the PI for the control Group A. This lengthening of the ttd from 4.6 days in

Figure 8.3 Challenge with *B. anthracis*.

Groups of 8-10 mice were challenged on day 0 with a SC injection of 0.25 ml of a diluted *B. anthracis* Vollum 1B spore preparation (369 cfu/ml). Thus each mouse was administered approximately 92 cfu which is equivalent to 14 LD_{50}. The mice were checked every 12 hours for 14 days.

The table opposite shows the mean time to death (ttd) for those animals which died in each Group and the percentage that died in each Group (%). A "protection index" (PI) was derived by the formula:

$$\frac{ttd}{\%dead} x100$$

The percentage of mice surviving in each group at each 12 hour interval after challenge are shown in the graph.

Group	No. in Group	Vaccinated with:
A	8	*S. typhimurium* SL 3261
B	8	PA + FIA
C	8	rPA + FIA
D	10	*S. typhimurium* SL 3261 / pORF1 + ampicillin
E	10	*S. typhimurium* SL 3261 / pORF1
F	10	*S. typhimurium* SL 3261 / pVET4m + ampicillin

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Anthrax Challenge

	A	B	C	D	E	F
ttd	4.6	5.2	4.5	6.2	5.5	6.4
%	100	75	62.5	70	100	90
PI	4.6	6.9	7.2	8.9	5.5	7.1

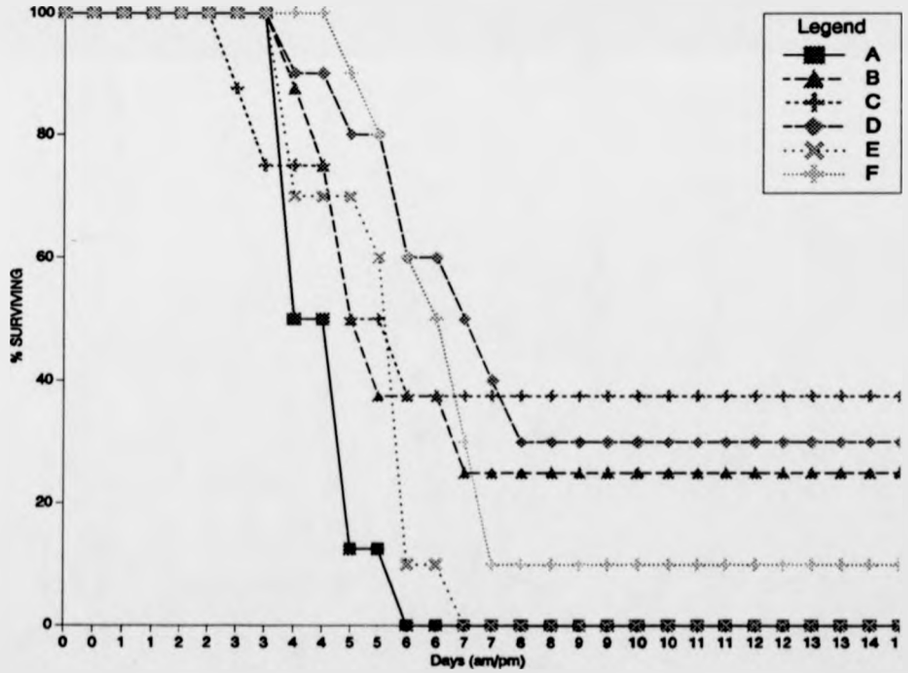

Figure 8.3 Challenge with *B. anthracis*

Figure 8.4 Histograms of deaths occurring in each challenged group per 12 hour period.

A *B. anthracis* Vollum 1B spore stock was prepared as described. All mice in groups A through F were challenged with a SC injection of 0.25 ml of a diluted spore preparation which was determined to contain 369 cfu/ml. Thus each mouse was administered approximately 92 cfu which is equivalent to 14 LD_{50}. There were 8 mice in Groups A, B and C and 10 mice in Groups D, E and F.

From the data in Figure 8.3 the number of mice which died in a given 12 hour period are plotted against time. There are two time points for each day to reflect the two 12 hour periods. All histograms show the same 3 to 8 day interval; no deaths occurred outside this interval.

Group	No. in Group	Vaccinated with:
A	8	*S. typhimurium* SL 3261
B	8	PA + FIA
C	8	rPA + FIA
D	10	*S. typhimurium* SL 3261 / pORF1 + ampicillin
E	10	*S. typhimurium* SL 3261 / pORF1
F	10	*S. typhimurium* SL 3261 / pVET4m + ampicillin

Facing page 167

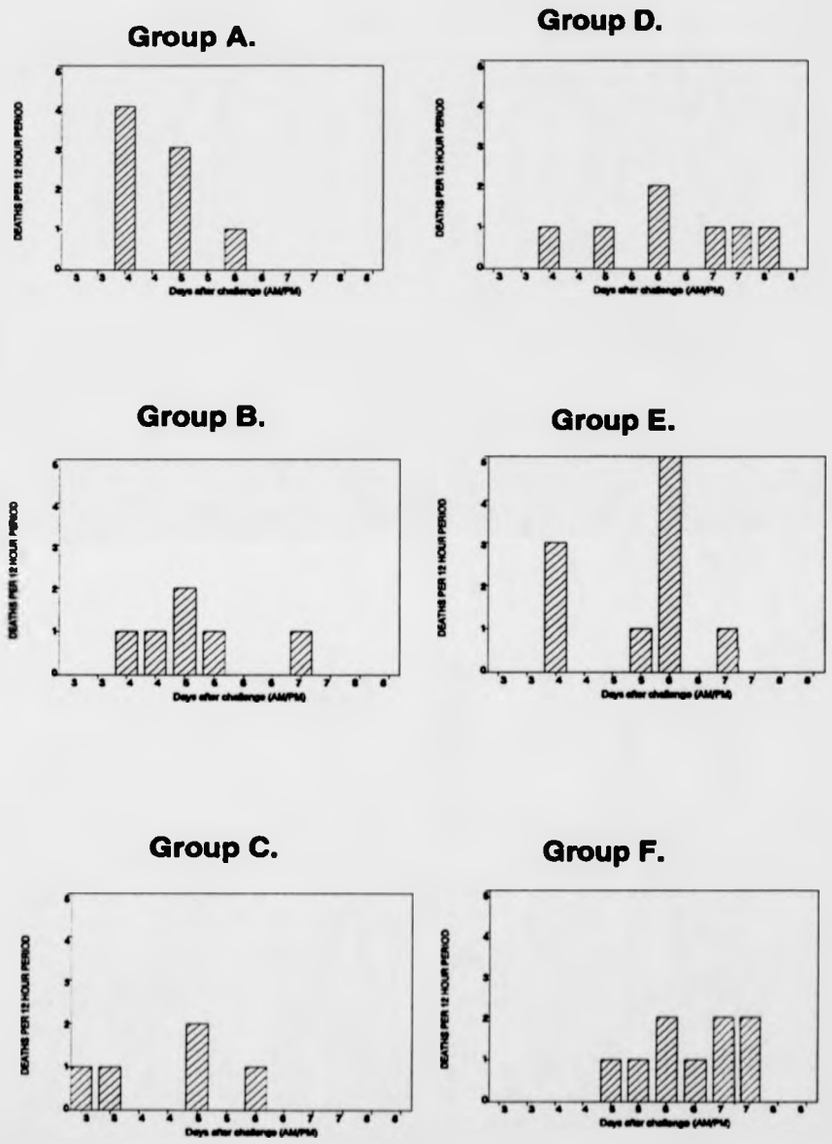

Figure 8.4 Histograms of deaths occurring in each challenged group per 12 hour period.

Group A may reflect a partial response. Although it is difficult to draw conclusions from limited numbers of animals, Group E may show a biphasic response (see Group E histogram, Figure 8.4) with 30% of animals dying on day 4 and the other 70% dying between days 5 to 7. This may reflect a non-responding group that dies early and a partially protected group.

In Group F, 1 animal (10%) survived with the deaths occurring between days 5 and 7. As with Group D, the ttd was lengthened at 6.4 and the PI was 7.1.

8.5.3 Mouse passage of *S. typhimurium* / pORF1 to increase colonisation level

Section 8.3.4 describes the colonisation levels of the clones *in vivo*. These were lower than expected compared to *S. typhimurium* SL 3261 and an experiment was conducted to assess if mouse-passaging the recombinant strains would increase the colonization level. In Group G, at day 14, an ampicillin resistant colony was isolated that was shown to still be expressing PA. This colony was designated 'G3c' and was grown in L-broth supplemented with ampicillin to make a seed culture. When this was used to inoculate 2 mice IV with $5x10^5$ bacteria (and with 7 days of SC ampicillin injections) it was found that at day 7 the organ count for the livers and spleens of these two mice were 10^4-10^5 *S. typhimurium* and most bacteria were still amp^r. Of the bacteria recovered, 20 of 20 colonies were still *aro*$^-$ and 9 of 9 were still expressing full-length PA. It is therefore possible to enhance the colonization level and this clone could be used in further animal experiments.

8.6 Conclusions

The initial experiment to establish the animal model was only partially successful as the wild-type *S. typhimurium* SL 1344 challenge dose was too low. It should have been greater so that all the control group died. The vaccinated group appeared to be protected from this challenge.

S. typhimurium infection can stimulate short-lived non-specific immune responses which can give protection against other infections such as *Listeria* but this protection is not apparent after 28 days (Killar & Eisenstein, 1985). It was not known if *S. typhimurium* infection could provide non-specific protection against *B. anthracis*, but precautions were taken to limit this possibility. Thus the mice were not challenged until 6 weeks after vaccination to allow the *S. typhimurium* to clear from the animals and non-specific immune

responses to diminish. The control animals (Group A), vaccinated with *S. typhimurium* SL 3261 alone, did not show any protective response to *B. anthracis* challenge at this time.

PA is produced by *B. anthracis* and secreted into the medium. The full-length rPA produced in *E. coli* containing pORF1 is exported into the periplasm. Western blotting suggested that denatured rPA behaved similarly to PA when reacting with both polyclonal and monoclonal antibodies. If antibody plays a role in protective immunity, neutralising antibody might only be induced when the PA was configured in the natural form so that conformational epitopes are present (Laver *et al.*, 1990). If rPA did not configure in this manner then the epitopes required to induce neutralising antibody might not be present. The results from Groups B and C show that both PA and rPA produce the same level of partial protective immunity. Although it is possible that the mechanism of the protective responses differ with the two antigens, it seems more likely that this result reflects the immunogenic similarity between the PA and rPA. The partial protection occurred in the presence of high anti-PA titres and confirms once again the antibody levels are not predictive of protection. If, as discussed below, protection can be T-cell mediated then the tertiary structure of the antigen may not be so critical anyway as only short peptides of PA would be presented on the surface of antigen presenting cells.

When *S. typhimurium* expressing PA are used to vaccinate mice they are partially protected against *B. anthracis* challenge. The *S. typhimurium* SL 3261 / pORF1 construct is very unstable and the phagemid is quickly lost. Phagemid instability is a problem identified by many workers (Charles & Dougan, 1990; Curtiss, 1990). Ampicillin treatment can stabilise pORF1 (Group D versus Group E) and this increases the level of protective immunity. The presence of pORF1 also leads to a much lower colonisation rate than *S. typhimurium* SL 3261 alone. This occurs even without selective pressure (Group E), and also with pBluescript alone and so it is not caused by PA expression. It may reflect a decreased ability of the bacteria carrying the phagemids to survive the initial clearance mechanisms of the body and to adapt to intracellular life. The initial inactivation or clearance of *S. typhimurium* is by macrophages (Collins, 1974) and to survive within the macrophage the *S. typhimurium* must activate gene transcription. This activation involves many proteins and can be detected within 30-60 minutes after macrophage infection (Buchmeier & Heffron, 1990; Alpuche Aranda *et al.*, 1992). It is possible that the presence of a high copy number plasmid could subvert cellular synthetic mechanisms from the rapid

induction of protein synthesis required for survival in the macrophage. In Section 7.3.1 it was shown that *S. typhimurium* SL 3261 containing pBluescript grew more slowly than *S. typhimurium* alone suggesting that the phagemid inhibited the bacteria in some way. An alternative possibility is that the manipulations (electroporation and selection *in vitro*) required to transform the bacteria with the phagemid select for strains of reduced virulence. This might involve changes in the LPS. Further work is needed to evaluate the mechanism of this effect. The LPS from the different strains could be examined for differences, also *S. typhimurium* / pBluescript could be cured of the phagemid and the *in vivo* colonisation of these two strains compared. This would show if the host *S. typhimurium* was altered by the manipulations used to transform it or whether the presence of the phagemid was required. If the manipulations to transform the bacteria lead to reduced virulence, then presumably this would be seen with all recombinant strains produced by these methods. This is not the case and so it seems more likely that it is an effect of the phagemid itself.

The immune responses occurred with a small number of bacteria and presumably with only a small amount of PA antigen being present. The fact that any immune response is produced with such a small antigen load reflects the effectiveness of this system of antigen presentation. The low level of organ colonization might be responsible for the production of only partial protection. It was possible to increase the colonization level of the recombinant by animal passage. *S. typhimurium* SL 3261 has been reported to have acquired additional attenuating lesions during laboratory passage as this strain still has reduced mouse virulence to its parent strain when the *aroA* lesion is complemented with a plasmid-borne *aroA* gene (Curtiss, 1990). If the process of electroporating any plasmid into *S. typhimurium* can select for attenuating lesions, such as LPS mutants, this process might be reversed by animal passage. Tarkka *et al.* (1989) have shown that the colonization rate of *S. typhimurium* SL 3261 containing a plasmid expressing an outer membrane protein of *Neisseria meningitidis* can be increased form 10^2 to 10^5-10^6 by a single mouse passage. Although the mutations that are acquired (or reversed) by this process are undefined, as long as the antigen is still expressed at the same level by the bacteria, then presumably more of the antigen is available to act as an immunogen. If protective responses are stronger this would suggest that research to increase the strain stability and level of expression would be worthwhile. The recombinant organism designated 'G3c' (Section 8.5.3) colonises at the

normal levels (10^3 times more bacteria), is stable with ampicillin selective pressure, and still produces PA. This might give significantly better immune responses.

The partial protection in Groups D and F occurs without ELISA detectable levels of anti-PA antibody. It is possible that the mechanisms of protection varies between the methods of antigen presentation. Although anti-PA antibody levels may not be predictive of protection, the antibody may have a role in protection when it is present. Thus in Groups B and C antibody and cell-mediated immunity (CMI) might combine to partially protect. It seems unlikely that the low levels of anti-PA antibody that might be present in the *S. typhimurium* vaccinated Groups are capable of partially protecting and so another immune mechanism is probably responsible. *S. typhimurium* are known to generate both $CD4^+$ and $CD8^+$ T-cells and these may play a role in protection against *B. anthracis*. To stimulate these cells the antigen is processed within antigen presenting cells (APC) into short peptides. These peptides are complexed with the major histocompatibility complex (MHC) I (for T_c-cells, $CD8^+$) and MHC II molecules (for T_h-cells, $CD4^+$) and presented on the surface of the APC where they are recognised by receptors on the appropriate T-cells (Germain, 1991). In this case the conformational structure of the antigen is not important and fragments of the antigen, that contain relevant T-cell epitopes, can induce protective responses. The cytoplasmic 63 kDa fragment of PA fused to a fragment of β-galactosidase expressed by pVET4m may not have the expected tertiary structure of PA, yet it can induce protective responses (Group F).

Further work would be needed to investigate the cells responsible for protective immunity and T-cell activation studies may elucidate the mechanisms of protection.

In conclusion, recombinant PA behaves in an immunologically similar manner to *B. anthracis* PA when used as an immunogen. Both can induce high anti-PA antibody titres but these titres do not correlate with protection and the animals are only partially protected. *S. typhimurium* containing pBluescript and phagemids derived from this vector colonise the liver and spleen at lower levels that *S. typhimurium* SL 3261 alone. These phagemids are unstable *in vivo* but can be partially stabilised by the concurrent administration of ampicillin. The level of colonisation can be improved by mouse-passage. Even at low colonisation levels *S. typhimurium* SL 3261 expressing PA can partially protect mice against SC challenge with a lethal dose of *B. anthracis* Vollum 1B. This protection occurs in the absence of ELISA detectable anti-PA antibody and may be due to the induction of

cell-mediated immune responses. *S. typhimurium* SL 3261 alone does not protect. These results demonstrate that PA expressed by *S. typhimurium* can induce protective immune responses in mice.

CHAPTER 9

Discussion

9.1 Discussion

Anthrax is still a significant animal and human pathogen. Current methods of control and vaccination are expensive and time-consuming and the vaccines used in humans are reactogenic and of limited efficacy. Experimental work has shown that a strong immune response to one component of the anthrax toxin, Protective Antigen, can protect against challenge (Ivins & Welkos, 1988). This allows a directed vaccine effort; with some diseases the protective antigens have not been identified, or a response to a number of antigens are required to stimulate protective immunity. Adjuvants that are approved for human use, when mixed with PA, are not able to stimulate strong protective immune responses even with multiple injections. The mechanism of protection against anthrax is not well understood, but cell-mediated responses are probably important (Ivins *et al.*, 1992). The aluminium adjuvants used in the current human vaccines do not stimulate strong CMI (Gupta *et al.*, 1993) and this may account for the lack of effectiveness of the human vaccines. If CMI is required, PA must be delivered in such a way as to generate this type of immunity. New adjuvants with the required capabilities are being developed but it is difficult to separate the adujvanting properties from the toxic side-effects (Gupta *et al.*, 1993). Live vaccine vectors are capable of delivering antigens to the immune system and stimulating various immune responses including CMI. Attenuated salmonellae are one such vector which offers this capability with the advantage of being orally active. PA could be expressed in this vector using recombinant DNA technology. The aim of this project was to develop a recombinant *S. typhimurium* that expressed PA and to evaluate the immune responses generated by this strain.

PA is poorly expressed from the native *B. anthracis* DNA sequences when these are cloned into *E. coli* (Vodkin & Leppla, 1983) and so the initial work concentrated on trying to improve expression. The effect on expression of moving the *B. anthracis* DNA insert from the intermediate copy number pBR322 vector into the high copy number pBluescript II was evaluated. It was found that there was a small increase in expression that was of the same order of magnitude as the increase in plasmid copy number between the two clones. Next the *B. anthracis* promoter was replaced with the *lac* promoter. This increased expression to some extent and suggested that the *B. anthracis* promoter had been limiting expression, but that another factor might now be limiting. An analysis of the promoter, RBS and predicted secondary mRNA structure failed to identify a problem, but an analysis of the

codon usage in the PA gene showed that this might be limiting translation. It was considered impractical to modify codon usage and so an alternative means to increase expression was considered. Although much is known about the regulation of transcription and translation the complexity of the interactions makes it difficult to predict the effect of changes in the DNA sequences. Until a more rigorous method of evaluating the interwoven effects of the DNA sequence is produced, constructs need to be made and tested.

The use of fusion proteins may increase expression as this uses the TIR of a characterised system. The 63 kDa C-terminal fragment of PA was selected as a candidate for expression as a fusion protein with part of β-galactosidase. β-galactosidase has been used as an antigen in *S. typhimurium* and generates appropriate responses (Brown *et al.*, 1987). The fusion protein was expressed at reasonable levels but seemed prone to proteolytic degradation. This susceptibility to degradation was shown to not be due to changes made to the C-terminal amino acid sequence. As the *lac* promoter is not a high level promoter, the 63 kDa fragment was expressed from the *trc* promoter. This construct produced only a small amount of full-length 63 kDa protein which from the appearance of smaller immunoreactive protein bands, appeared unstable. The full-length PA was not degraded in this fashion. This could be due to its cellular location or to the presence of the 20 kDa N-terminal fragment. The full-length PA was expected to be periplasmically located. The 20 kDa fragment is not required for the biological activity of PA, but presumably has another function (Leppla, 1991). It might stabilise the PA and prevent proteolytic degradation or it could be required for the correct secretion and folding of PA. Gram-positive secreted proteases have a similar propeptide sequence (Wandersman, 1989). It has also been postulated that it is required to keep the 63 kDa fragment in a soluble form and prevent this hydrophobic protein from interacting with cell membranes during processing (Leppla, 1991). It was decided to investigate the cellular location of the expressed PA and the effect of the 20 kDa fragment.

The cellular location of the full-length PA expressed from phagemid pORF1 (which followed the *B. anthracis* signal sequence) was primarily in the periplasm. The 63 kDa fusion protein from pVET4m was located cytoplasmically and was less stable. An attempt to secrete this 63 kDa fusion protein into the periplasm with the LT-B signal sequence did not work. This may have been due to the protein fragment failing to pass through the

transport mechanism and becoming trapped in the membrane. This suggests that the 20 kDa fragment (or other fusion protein) may be needed for correct export of the PA protein.

Although none of the recombinant strains are suitable vaccine candidates, as they express low levels of PA, are unstable and contain an ampicillin marker, they were used to show if protective immune responses against *B. anthracis* challenge could be generated by PA expressed in *S. typhimurium*. The recombinant organisms were evaluated to select the most suitable for study. Their stability *in vitro* was measured as an indicator of *in vivo* stability. It was found that many of the clones were unstable without ampicillin selection present. Subsequently it was found that the *in vitro* results did not correlate well with the *in vivo* results, as was also noted by Molina & Parker (1990). When *Salmonella* invade the host cells and subsequently enter macrophages, they must rapidly express many different bacterial proteins to survive in the changing environment (Finlay *et al.*, 1989). This ability to rapidly respond to external conditions is not tested during *in vitro* growth. An attempt to stabilise one of the phagemids with the *cer* region was not successful. There are many mechanisms that might lead to plasmid instability and the *cer* region would only effect stability if formation of multimers was the cause of plasmid loss (Summers & Sherratt, 1984). pBluescript was probably an inappropriate vector to use; it contains the intergenic region of the f1 phage which has no function in a *S. typhimurium* vaccine vector and may impose additional negative selection pressure on phagemid stability. High copy number vectors can be unstable and reducing the copy number can increase the stability (Salas-Vidal *et al.*, 1990). Thus the use of a lower copy number vector, such as pBR322, could improve stability. From all the available data two constructs, pORF1 and pVET4m, were chosen for animal work. The choice of two constructs allowed a comparison between a periplasmically located full-length product and a cytoplasmically located fusion between β-galactosidase and the 63 kDa C-terminal fragment.

The vaccination of mice with PA, rPA and with the two *S. typhimurium* recombinant strains and their subsequent challenge with virulent *B. anthracis* was described. Recombinant PA was shown to induce partial protection comparable to native PA when both were purified and administered with adjuvant. This showed that the expressed rPA behaved in a similar immunological fashion to native PA. As it was known that the plasmids were unstable they were given IV and with daily ampicillin injections. Ampicillin was shown to enhance stability of the plasmids *in vivo*. The PA expressing *S. typhimurium* only colonised

mice at low levels. *S. typhimurium* containing only pBluescript also colonised at intermediate levels and the possible reasons were discussed. *In vitro* growth curves suggested that pBluescript alone could slow the growth of *S. typhimurium* SL 3261.

A mouse passaged strain (Section 8.5.3) was shown to colonise at levels equivalent to *S. typhimurium* and could be used in future studies. The mechanism of this change is unclear and could reflect an alteration in the host bacteria or a mutation in the phagemid. This could be resolved by recovering the phagemid and electroporating it back into the original stock of *S. typhimurium* SL 3261 and also curing the mouse passaged strain of pG3c and electroporating pORF1 into the host. Both constructs could then be tested to identify if the ability to colonise at the higher level is conferred by the phagemid or host.

Although the *S. typhimurium* expressing PA only colonised at low levels (Group G colonised below 10^3 cfu/organ against 10^5 for the carrier strain) they still managed to induce partial protective responses (30% survived in Group D). These protective responses occurred without detectable anti-PA antibody. Protective responses in the absence of detectable antibody have also been reported for *S. typhimurium* expressing the Circumsporozoite protein of mouse malaria (Flynn *et al.*, 1990) and the P.69 antigen of *Bordetella pertussis* (Strugnell *et al.*, 1992). Although it is possible that a small amount of high affinity antibody could have been undetected yet responsible for protection, it seems more likely that CMI was generated by the constructs and contributed to protection. Only partial protection was obtained but this may be due to the low colonisation levels failing to provide sufficient quantity of antigen to the immune system to generate strong responses. Also ampicillin was only administered for 7 days after the two IV injections and this regimen was shown to only partially stabilise the phagemid and the phagemids were lost at a higher rate once the antibiotic was stopped. Without ampicillin (Groups E and H) the phagemid was rapidly lost and all mice died when challenged. A construct that colonised at high levels and produced a high level of PA over the full period of bacterial persistence would be expected to induce better protection. The mouse-passaged phagemid pG3c may induce a higher level of protection by presenting a larger mass of antigen by virtue of its higher colonisation level.

In summary, this work has investigated the problems of stable high-level expression of the PA of *B. anthracis* in *S. typhimurium*. Although the expression level was increased by changing the promoter, it appeared that other factors were limiting expression. The importance of the 20 kDa N-terminal fragment for export was shown and the problems of

exporting fragments of the PA identified. Although a candidate vaccine strain was not produced in this work it has shown where the problems are and provides a basis for further expression studies. It was shown that in principal PA expressed by *S. typhimurium* can induce protective responses, even when only low colonisation occurs. This system may also help identify the mechanism of protection against anthrax. These results show the potential for this approach and suggests that further work to stabilise and increase the expression of PA would be worthwhile.

Various general strategies are available for expression of toxic or unstable antigens and some of these are shown in Table 9.1. An antigen can be expressed in an insoluble form within the cytoplasm or it can be exported from the cytoplasm where it may be more stable (Enfors, 1992). The host strain may influence stability and protease negative mutants may degrade foreign proteins less. Although the full-length native protein may be required for some expression studies, for vaccine work it may be possible to modify the antigen so that it is no longer toxic or unstable yet still immunogenic (Curtiss, 1990). Many different systems are available for this type of protein engineering and some have been discussed in Chapter 5.

Plasmid expression offers the possibility of multiple gene copies but the plasmids can be unstable and often contain antibiotic markers. The problem of antibiotic markers can be overcome to some extent by the use of balanced lethal mutations and various systems have been described (Nakayama *et al.*, 1988; Fulginiti *et al.*, 1992). If plasmids are used they must be stable and this work has suggested that pBluescript was not suitable as it was found to impair colonisation levels itself. Other plasmids may be more successful. If plasmid expression is still unsuitable, the gene can be integrated into the chromosome and this can be coupled with the introduction of further attenuating lesions (Hone *et al.*, 1988b; Strugnell *et al.*, 1990). Only a single copy is usually chromosomally integrated and so a suitable promoter that produces sufficient protein must be used.

There are many classes of promoters that can be used in both plasmids and chromosomal integration. If a protein is toxic then high level constitutive expression may be impossible. Alternatively a promoter can be used which only induces expression in particular environments; especially the antigen presenting macrophage. This allows the bacteria to colonise at normal levels and only then is expression switched on. Even if bacterial death occurs, due to the toxic nature of the antigen, the antigen will be presented

System	Comments	References
Engineer antigen, or its form or location, so not toxic to the cell.	Express antigen as fragments or just express epitopes. Can alter cellular compartment of antigen or express as fusions or insoluble inclusions.	Charles & Dougan, 1990; Curtiss, 1990; Stocker, 1990; Enfors, 1992.
Select different carrier strain.	Different strains may degrade antigens differently, eg *htrA* mutants.	Johnson *et al.*, 1991; Chatfield *et al.*, 1992; Strahan *et al.*, 1992.
Chromosomal integration to stabilise inserted DNA.	Stabilises gene but single copy number.	Hone *et al.*, 1988b; Strugnell *et al.*, 1990; Flynn *et al.*, 1990.
Inducible promoters express antigen *in vivo*.	Use of anaerobic or macrophage inducible promoters to drive expression in particular environments.	Buchmeier & Heffron, 1990; Oxer *et al.*, 1991; Chatfield *et al.*, 1992.
Balanced lethal mutations to stabilise plasmids.	*asd* or *pur* on plasmid complements deleted gene in chromosome.	Nakayama *et al.*, 1988; Fulginiti *et al.*, 1992.
Inverted promoters.	Only a proportion of cell switch on expression at any one time.	Yan *et al.*, 1990.
Repressor on incompatible plasmid.	Repressor lost *in vivo* as plasmid free segregants occur and so only a proportion of cells constitutively express antigen.	Ervin *et al.*, 1992.

Table 9.1 Systems for stable expression of antigens

to the immune system by the macrophage. Expression may result in the death of the bacteria and so the persistence phase of *S. typhimurium* colonisation may be lost. The level of the immune response generated is related to the persistence phase and so this may moderate the response. In this case the bacteria may function more as an antigen delivery vehicle than as a living vaccine with a persistence phase and long term antigen presentation. Alternative promoter systems achieve a similar effect by ensuring that only a proportion of the bacteria switch to expressing the antigen to high levels at any one time and so if this induces bacterial death the non-expressing bacteria survive and persist (Yan *et al.*, 1990; Ervin *et al.*, 1992).

With regard to PA, the most successful expression was achieved by exporting the full-length product into the periplasm. The instability of the phagemid and low colonisation levels of the bacteria may be due to the pBluescript vector used. The *lac* promoter could be replaced with a stronger promoter to identify if this is limiting expression in the full-length construct (the *trc* promoter was only used with the 63 kDa fragment). If this has little effect then other factors, such as codon usage, may be important. Replacing the coding sequence of the similarly AT rich tetanus toxin C-fragment with a sequence optimized for *E. coli* codon usage increased expression levels (Makoff *et al.*, 1989). The level of expression could also be assessed in periplasmic protease mutants (such as *htrA* (Johnson *et al.*, 1991; Chatfield *et al.*, 1992)) as these may degrade secreted proteins less than wild-type strains. Alternatively, efforts could be made to express PA as an insoluble fusion protein within cytoplasmic inclusion bodies. This work suggests that the 63 kDa fragment of PA may not be a suitable candidate for expression, but other fragments might be both stably expressed and immunogenic. Protective epitopes have not yet been identified for PA and so it would be necessary to clone different fragments of PA and assess both expression and their protective effect. Until the mechanism of protection against *B. anthracis* is established, and a test devised which correlates with protection, it will be necessary to test such constructs for their ability to induce protective immunity. Inducible promoters, such as the anaerobic promoter *nirB* or macrophage inducible promoters, could help stabilise a plasmid as the protein would only be expressed after bacterial invasion in an immunogenic site. If the plasmids are still unstable, chromosomal integration could be considered although a suitable high level promoter will be needed.

The results obtained in this study suggest that protection against challenge with *B. anthracis* can occur in the absence of detectable anti-PA IgG antibody. *S. typhimurium* is known to induce CMI and the relevance of this to protection could be studied. An initial approach would be to carry out T-cell proliferation assays on spleen cells from vaccinated animals. If it can be shown that T-cells proliferate in response to PA, then the cell class involved can be identified. Their relevance to protection could be shown by depleting the identified T-cell class in vaccinated mice using specific monoclonal antibodies and seeing if protection was impaired. Additionally it may be possible to show that a particular T-cell clone can adoptively protect unvaccinated mice.

In conclusion, this work has identified a promising approach to developing a new anthrax vaccine. *S. typhimurium* expressing PA can induce protective responses against challenge with *B. anthracis* spores. Although work is needed to increase the expression level of PA, stabilise the inserted genetic material and ensure suitable colonisation levels these problems should not be insurmountable and various approaches to solving them have been identified. With further work, the goal of developing an oral vaccine is achievable.

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P' = promoter

***PA* = Native PA promoter**

***lac* = *E.coli* promoter**

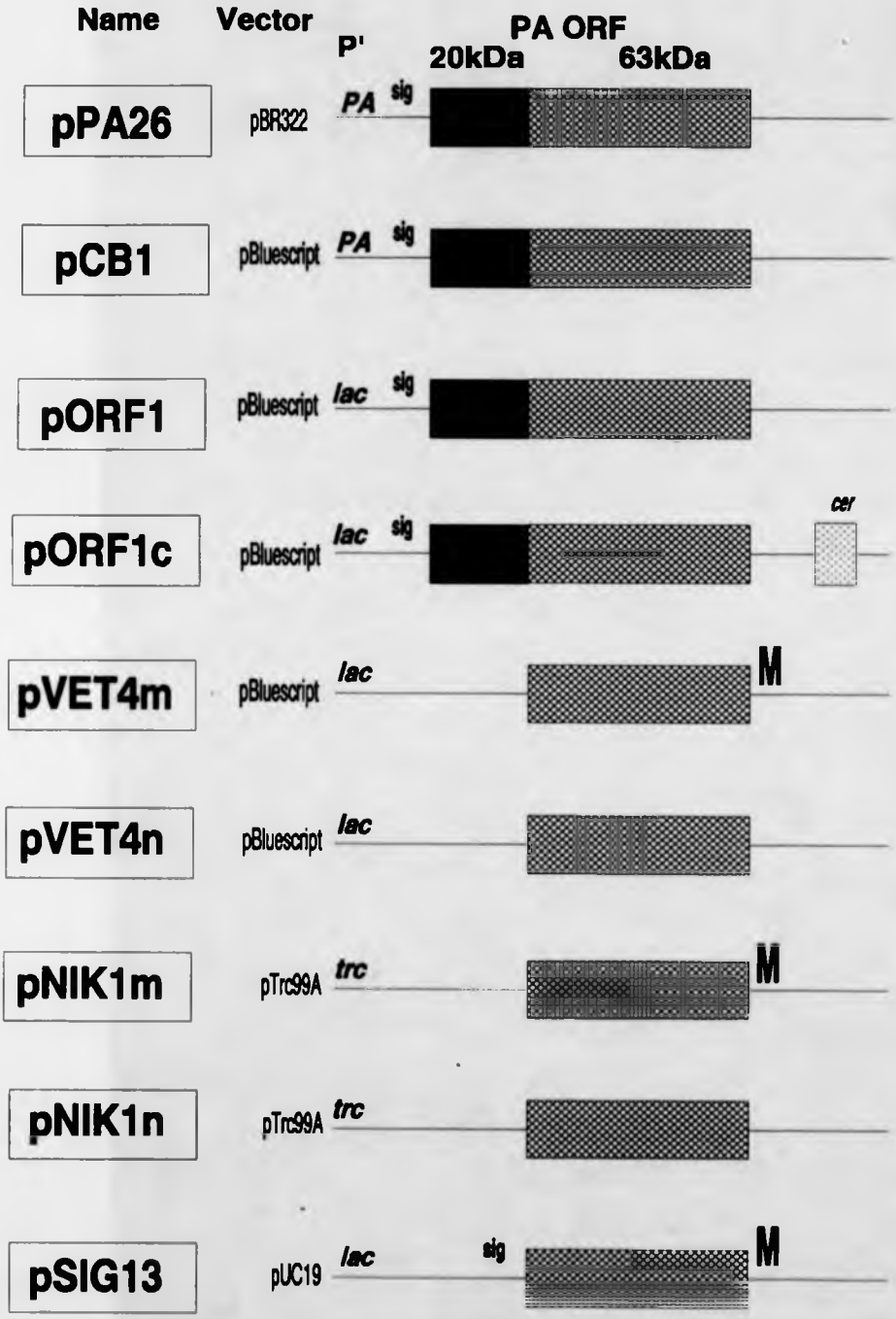
***trc* = hybrid promoter**

sig = signal sequence,

Native PA signal sequence in all but plasmid pSIG13 which has the *E. coli* LT-B signal sequence.

***cer* = partioning region from ColE1**

M = modified C-terminus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